EXPLORING THE MULTIPLE FUNCTIONS OF NEUTROPHILS

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by

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Neutrophils are one of the most effective antimicrobial cells in the innate immune system. They arrive at a site of infection or inflammation in response to a chemotactic gradient. At this site, they phagocytose invading pathogens and aid in their destruction through both oxidative and non-oxidative mechanisms. This is known to be mediated through the release of toxic granule contents and antimicrobial effector proteins. After their antimicrobial activity is performed, neutrophils undergo a programmed cell death process known as apoptosis and are quickly cleared from sites of inflammation by phagocytes. As such, neutrophils are believed to be fast-acting, short-living effector cells that perform antimicrobial activities with limited influence on ensuing immune responses.

Nevertheless, neutrophils are increasingly being shown to be regulators of adaptive immune responses. I show that neutrophils can act as professional antigen presenting cells capable of processing and presenting antigen to T cells. I also show that acquisition of antigen presentation ability is T cell-contact dependent and that the resulting T cell response is skewed towards a T_h1 and T_h17 phenotype.

Neutrophils also have the ability to undergo a cell death process termed NETosis in which they extrude extracellular DNA that can trap and kill pathogens. I show that both mouse and human

neutrophils undergo NETosis in response to the protozoan parasite *Toxoplasma gondii* and that the resulting entrapment leads to parasite killing. NETosis in response to *Toxoplasma* is parasite-invasion independent and is mediated partly by signaling through the ERK pathway.

The in vivo role of neutrophils during a *Toxoplasma* infection was also addressed. I show that neutrophil depleted and infected mice succumb to infection with highly dysregulated pulmonary immune responses. Depletion of neutrophils led to an increased phagocyte inflammatory state that resulted in an increase in T cell activity and cytokine production leading to pathology and ultimately death of the host.

Data presented in this thesis challenge the traditional view of neutrophils and portray them more as immunoregulatory cells that can influence, shape, and regulate an immune response well beyond the stages of innate immunity and even beyond their own death.

BIOGRAPHICAL SKETCH

Delbert was born on December 1st, 1982 to Darlene and Sami Abi Abdallah in Beirut, Lebanon. Along with his two older brothers and younger sister, he led a very active childhood and loved to play outdoors. As for science, he developed a strong interest at an early age and was fascinated by all forms of biology. Delbert would venture into the woods surrounding his family home and spend hours exploring the wilderness and collecting various small-sized animal specimens which are still preserved in formalin at the family home. He attended Saint Joseph School, Cornet Chehwan for his pre-college education and had an extreme interest in science throughout his high school years. He started and participated in many science clubs and participated in and won many science competitions. To pursue a higher education degree, Delbert left his native country of Lebanon and moved to the United States of America. In keeping with his scientific interests, Delbert attended the University of Florida (UF) in Gainesville, Florida for his undergraduate studies and graduated with a Bachelor of Science degree in Microbiology and Cell Science with a minor in Chemistry. At UF Delbert was a member of Dr. Peter Kima's laboratory and studied the parasite *Leishmania.* His years spent in the Kima laboratory solidified his interest in pursuing a career in science and were instrumental in his decision to apply to graduate school and pursue a Ph.D. In 2006, Delbert matriculated at Cornell University in the interest of obtaining a Ph.D in immunology where he joined Dr. Eric Denkers' laboratory. Delbert is an extremely social person who enjoys the company of family and friends. He leads an outdoors-heavy lifestyle and enjoys hiking and fishing. Delbert is also an avid backgammon player and has a keen interest in politics, reading and Middle Eastern music.

I dedicate this dissertation to my family:

Darlene, Sami, David, Dennis and Daisy

who have never failed to always be there for me

&

to all the animals that have given

their lives to make this work possible

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I also would like to acknowledge Dr. Barbara Butcher for her constant mentoring and guidance in the laboratory and outside it. She has always provided me with great tips and insights for many experiments, and on many occasions helped with the planning and execution of experiments as well. She has also been a great friend and a great person to spend time with and talk to. I also would like to thank Dr. Charlotte Egan for her un-ending support and constant help with experimental work. She is a fantastic colleague to work with and has always been available for suggestions and help, especially when needed. She is also a good role model who has always pushed me to go the extra step and achieve my potential. She is also a great friend and an extreme pleasure to work with. I also would like to thank members of the Denkers laboratory who have made my graduate studies a much more pleasant experience and for constantly providing insight and useful commentary. In that regard, I want to thank Dr. Kirk Maurer, Dr. Anne Schneider, Ms. Sara Cohen, Dr. Ali Bierly, Dr. Woraporn Sukhumavasi, Dr. Jin Leng, Dr. Chiang Lee and Mr. Mozammal Hossein. They have been great colleagues to work with and have had a positive effect on my graduate career. A special thank you goes to Dr. Norah Smith and Ms. Cohen for being great friends and colleagues. I also want to thank my committee members, Dr. Margaret Bynoe and Dr. Susana Mendez for constant support and guidance and Dr. Scott Coonrod for agreeing to sit on my committee as an external examiner.

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LIST OF ABBREVIATIONS

ACAMP, apoptotic-cell-associated molecular pattern
AIDS, acquired immune deficiency syndrome
AML, acute myeloid leukemia
APC, allophycocyanin or antigen presenting cell
BALF, bronchoalveolar lavage fluid
BC, band cell
BPI, bacterial permeability-increasing protein
BSA, bovine serum albumin
CCR, chemokine (C-C motif) receptor
CD, cluster of differentiation
CEACAM, carcinoembryonic antigen-related cell adhesion molecule
CFSE, 5-(and 6-)carboxyfluorescein diacetate succinimidyl ester
CGD, chronic granulomatous disease
CR1, complement receptor 1
CXCL, chemokine (C-X-C motif) ligand
DAPI, 4′,5-diamidino-2-phenylindoleI
DC, dendritic cells
DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DMSO, dimethylsulfoxide
DNA, deoxyribonucleic acid
EDTA, ethylenediaminetetraacetic acid
ELISA, enzyme-linked immunosorbent assay
FACS, fluorescence-activated cell sorter
FCS, fetal calf serum
FITC, fluorescein isothiocyanate
fMLF, formylmethionyl-leucyl-phenylalanine
GAPDH, glyceraldehyde 3-phosphate dehydrogenase
GFP, green fluorescence protein
GM-CSF, granulocyte-monocyte colony-stimulatory factor
H&E, hematoxylin and eosin
hCAP, human cathelicidin antimicrobial protein
HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HNP, human neutrophil peptide
i.p, intraperitoneal
ICAM, inter-cellular adhesion molecule
IFN-γ, interferon gamma
IgG, immunoglobulin G
IGTP, IFN-γ-inducible GTP-binding protein
IL, interleukin
IRG, immunity-related GTPases
KO, knockout
LPS, lipopolysaccharide
mAb, monoclonal antibody

MAC-1, macrophage 1 antigen
MACS, magnetic-activated cell sorting
MAPK, mitogen-activated protein kinase
MB, myeloblast
MBL, mannose-binding lectin
MC, myelocyte
MCP, macrophage/monocyte chemotactic protein
MHC, major histocompatibility complex
MIP, macrophage inflammatory protein
MLN, mesenteric lymph node
MM, metamyelocyte
MMP, matrix metalloproteinase
MPO, myeloperoxidase
mRNA, messenger ribonucleic acid
MyD88, myeloid differentiation primary response gene 88
NADPH, nicotinamide adenine dinucleotide phosphate-oxidase
NET, neutrophil extracellular trap
NGAL, neutrophil gelatinase-associated lipocalin
NK, natural killer
NO, nitric oxide
OCT, optimal cutting temperature
OVA, ovalbumin
PAD, peptidylarginine deiminase
PBS, phosphate buffered saline
PCR, polymerase chain reaction
PE, phycoerythrin
PEC, peritoneal exudate cells
PGRP, peptidoglycan recognition proteins
PM, promyelocyte
PMA, phorbol 12-myristate 13-acetate
PMN, polymorphonuclear leukocytes
PS, phosphatidylserine
PSGL-1, P-selectin glycoprotein ligand 1
PV, parasitophorous vacuole
ROS, reactive oxygen species
SCID, severe combined immunodeficiency
SLE, systemic lupus erythematosis
SPL, spleen
STAg, soluble tachyzoite antigen
TCR, T cell receptor
TGF, transforming growth factor
TLN, tracheobronchial lymph node
TNF, tumor necrosis factor
WT, wild type

LIST OF SYMBOLS

α: alpha
β: beta
°: degrees
ε: epsilon
γ: gamma
μ: micro
Φ: theta or phage

CHAPTER 1

INTRODUCTION

Toxoplasma

Discovery, history and life cycle of *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular protozoan parasite belonging to the phylum Apicomplexa, subclass coccidian (1). It is a ubiquitous parasite that was discovered about 100 years ago by two different independent research groups. Charles Nicolle and Louis Manceaux discovered *Toxoplasma* in 1908 in tissues of a North African hamster-like rodent (*Ctenodactylus gundi*) in Tunis, while Alfonso Splendore discovered it, also in 1908, in a rabbit in Brazil (2, 3). The parasite has since been found to infect most if not all warm-blooded animals and has been described as one of the most successful parasitic organisms worldwide (4).

The parasite has the ability to infect a wide range of intermediate hosts, but it was not until 1965 that its definitive host was identified as the cat (5). A few years later, in 1970, the definitive host cycle was elucidated following discoveries that the sexual development stages occurred in the small intestine of the cat (4, 6, 7). The infectious agents of *T. gondii* for all hosts have been defined to belong to either one of the following life cycle stages: tachyzoites, bradyzoites (contained in tissue cysts) or sporozoites (contained in oocysts). After ingestion of tissue cysts or oocysts, the bradyzoites or sporozoites transform into rapidly dividing tachyzoites that can invade and infect virtually any nucleated cell. The tachyzoite is most often seen as crescent shaped and will enter a host cell through a process that involves active invasion and will divide within a host cell by multiplying asexually by repeated endodyogeny until the host cell runs out of intracellular space and ruptures as a result. Host cell invasion by toxoplasma is parasite mediated and once inside the cell, the parasite resides in a parasitophorous vacuole (PV) that is

protected from host cell killing mechanisms. The PV is formed by invagination of the host cell membrane but at the same time excludes most transmembrane proteins (8, 9). The PV has a nonfusigenic nature and thus it remains segregated from both the exocytic and endocytic pathways of the host cell (10). This active invasion is essential for survival of the parasite since phagocytosis of antibody opsonized parasites leads to the incorporation of the parasite into the phagosomal maturation pathway eventually killing the parasite (11). After a number of divisions occur, a tachyzoite will give rise to a tissue cyst full of bradyzoites that is characterized by its dormant nature in muscular and neural tissues. These cysts are usually harmless and can persist for the life of the host and can be found in almost all intermediate hosts (12). Oocysts are shed by cats after ingesting any of the three infectious stages of *T. gondii*. The most common route of infection in cats is the ingestion of tissue cysts, after which the cyst wall is broken down by enzymes in the stomach and small intestine, where bradyzoites are released and infect small intestine epithelial cells (13). Five distinct morphological stages of *T. gondii*, defined as types A to E schizonts, occur before the parasite enters its sexual stages to produce oocysts (14). Felines shed *Toxoplasma* in their feces in the form of non-infective, unsporulated oocysts that become sporulated and infective in the environment. Each sporulated oocyst contains 2 elliptically shaped sporocysts, each containing 4 sporozoites. Feline shed oocysts are extremely resistant to harsh environmental conditions and are able to survive in fresh/seawater, soil, and can persist even after chemical and physical treatment of contaminated water including chlorination, ultraviolet rays and ozone treatment (refer to Figure 1.1 for a life cycle) (15, 16).

Toxoplasmosis in humans and animals

T. gondii is the causative agent for the disease Toxoplasmosis and is one of the most prevalent protozoan parasitic infectious agents in man and mammals. It is observed that the seroprevalence rates for both humans and animals can vary widely between geographic locations dues to various factors including but not limited to food consumption behaviors, food production practices, governmental control and safety procedures as well as animal-related hygiene. In humans, seroprevalence can be as high as 16-40 % in the Unites States, and 50-80 % in South America and continental Europe (17). It is important to note that the seroprevalence in humans has been steadily declining over the past few decades (18, 19).

In humans, acute *T. gondii* infection is mostly asymptomatic in immunocompetent adults and children but can cause severe disease in immunocompromised hosts, most notably in individuals with Acquired Immune Deficiency Syndrome (AIDS). The most common clinical manifestation of *T. gondii* in AIDS patients is encephalitis due to the reactivation of the parasite during latent infections (20). In the United States, anywhere from 10% to 40% of adults with AIDS are latently infected with *T. gondii* and about one third of these patients will develop toxoplasmic encephalitis (21). *Toxoplasma* infection also poses a serious risk for fetuses if the infection is acquired during gestation. Depending upon the stage of pregnancy, congenital infection can have different subclinical or clinically apparent outcomes. Infections occurring in the first trimester are generally more severe than infections acquired in the second or third trimester, while infections acquired before gestation normally pose little or no risk at all (1, 22). Infection during gestation can result in visual and hearing losses, mental retardation and sometimes death, with

the most common manifestation of infection being ocular disease (23). The major sources of parasite in postnatally acquired infections is through the ingestion of tissue cysts present in undercooked meat or by consumption of contaminated water or food by oocysts shed in cat feces (17). Another rare but important source of infection is by organ transplantation or blood transfusions between an infected and a non-infected individual (1, 24). These types of infected individuals are at high risk of cyst recrudescence due to the fact that they are under an immunosuppressive regimen of drugs. One also has to take into consideration the potential for possible infection with the parasite in laboratory personnel due to the exposure to infected needles, glassware or even animals.

In addition to its relevance in humans, *Toxoplasma* has been found to infect a wide range of animals ranging from domestic animals, wild animals, fur-bearing carnivores, wild felids, wild pigs, sea mammals, monkeys, marsupials, ungulates and even birds (25). The parasite is known to cause major agricultural economic losses especially in goats and sheep where millions of lambs are lost yearly throughout the world due to *Toxoplasma* induced abortions (26). The emergence of infections in marine mammals is a more recent development in the epidemiology of *Toxoplasma* where reports are surfacing of infections in otters, seals, sea lions and dolphins (27-29). It is postulated that coastal pollution by run-off from contaminated freshwater or sewage as a result of human or pet activity might be the source of *Toxoplasma* infections in marine mammals. As a result, filtration of water by shellfish can take place leading to a highly concentrated amount of oocysts in these shellfish that in turn become a source of infection in the marine environment (25, 30).

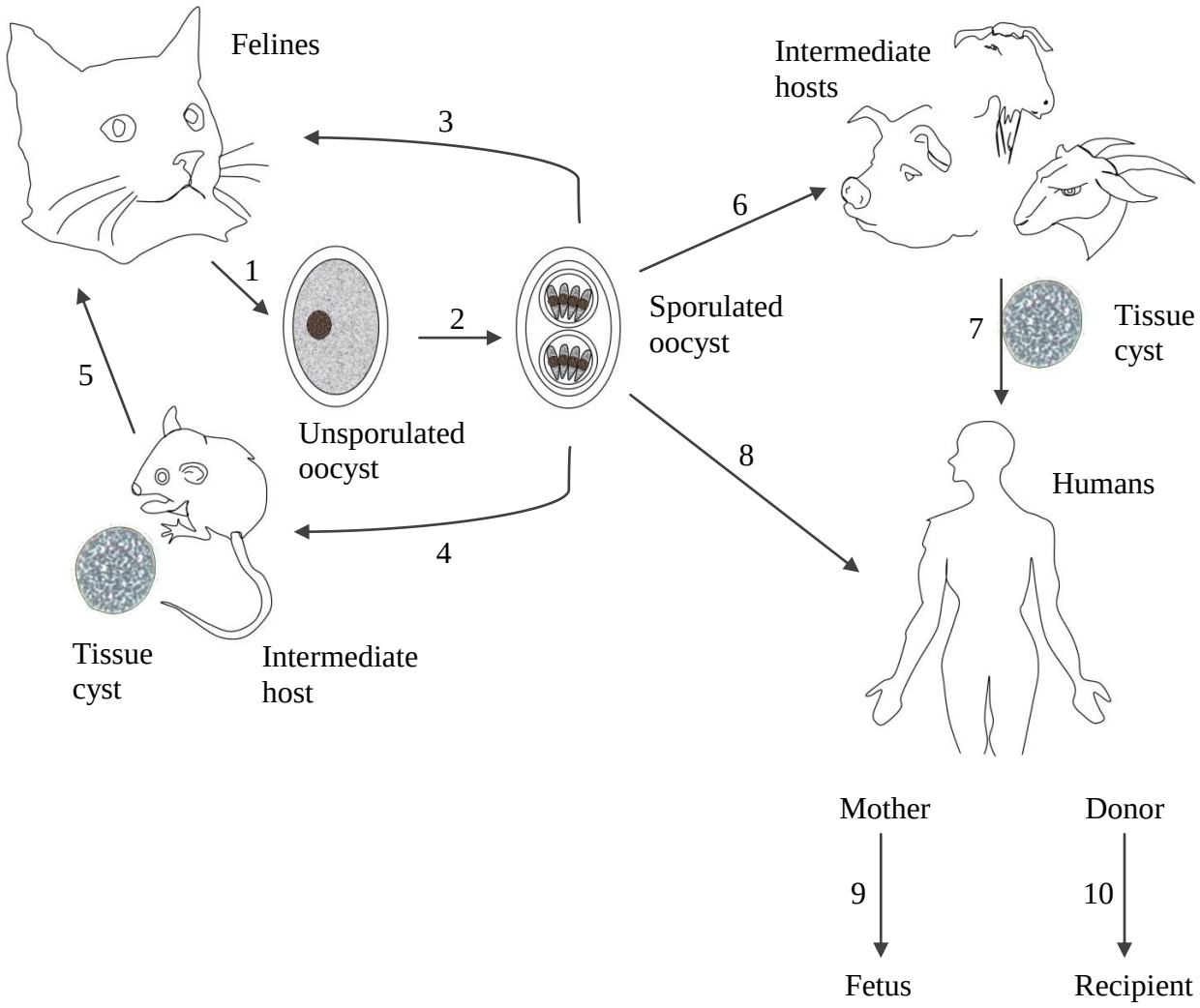


Figure 1.1 ***Toxoplasma gondii*** **life cycle.** Cats shed unsporulated oocysts in their feces (1) which can sporulate and become infective in the environment (2). Cats can then be re-infected by ingestion of sporulated oocysts (3). Cats can also ingest tissues cysts (5) found in intermediate host that had ingested sporulated oocysts (4). Also, humans may be infected by ingesting sporulated oocysts (8) or tissues cysts (7) from infected intermediated hosts that had ingested sporulated oocysts (6). Infected pregnant women can infect their fetuses through transplacental infections (9) and *Toxoplasma*-negative blood or organ recipients can get infected with the parasite if their donor is *Toxoplasma*-positive (10).

Diagnosis, treatment and prevention of *T. gondii*

Various techniques have been developed over the years to aid in the determination of whether an individual or a particular animal is positive for *Toxoplasma* or not. *T. gondii* can be directly detected in tissues of infected hosts or indirectly diagnosed using serological methods. The Sabin and Feldman dye test was developed in 1948 to detect *Toxoplasma* antibodies in patient sera that can react with tachyzoites (31). Since then, other methods have been developed to diagnose seropositivity such as ELISA, IgG avidity test and agglutination (32-35). Parasites can also be directly detected in tissues through simple immunohistochemical procedures using widely available anti-toxoplasma antibodies or even a simple Giemsa stain or through PCR of the highly repetitive B1 *Toxoplasma* gene (36).

Guidelines for the treatment of *T. gondii* infection have been established over the years. The regimen of drugs most often prescribed entails a combination of pyrimethamine, sulfadiazine and folinic acid (37). This regimen can vary depending on the infected host and stage of infection. For example, acute toxoplasmosis in women is often treated with spiramycin in the first trimester. AIDS patients with acute toxoplasmic encephalitis are sometimes treated with clindamycin instead of sulfadiazine. However, a regimen of pyrimethamine and sulfadiazine supplemented with folinic acid remains the gold standard for non-difficult cases and is administered for the duration of clinical symptoms and is continued for 1-2 weeks after the resolution of symptoms (1). It is important to note that this combination of drugs is only effective against the actively dividing, acute stage of infection and normally have no bearing on the dormant, chronic phase of toxoplasmosis (22).

Various preventative measures can be taken to inhibit infection in humans and animals of agricultural importance. Certain countries undertake a systematic screening of all pregnant women to minimize congenital infections and some advocate that pregnant women should be made aware of the dangers of congenital toxoplasmosis through routine obstetric care (1, 38). Simple hand washing and cooking techniques should be employed to effectively minimize human infections. Most infective stages of *T. gondii* are killed by exposure to extreme temperatures (hot or cold) and tissues cysts are also documented to be killed by gamma irradiation (22, 39). An effective vaccine against *T. gondii* licensed for human use has not been developed yet, however a vaccine against *Toxoplasma* in sheep has been developed using the attenuated live S48 strain of the parasite (40). Recent efforts in this regard have focused on vaccine candidates that can mimic the naturally occurring T_h1 and humoral responses normally seen in humans after natural infections (1). Approaches have included using purified or recombinant parasite surface antigens, parasite secreted proteins (SAG-1/p30, MIC3), DNA vaccines and live attenuated or mutant forms of the parasite (CPS-1) (41-44).

Taking into consideration all of the health hazards posed by the parasite and given its widespread prevalence in both the animal and human populations, it is vital to study the immune response to the parasite and to better understand its mechanism of disease induction in order to better formulate effective preventative and treatment measures, especially in humans.

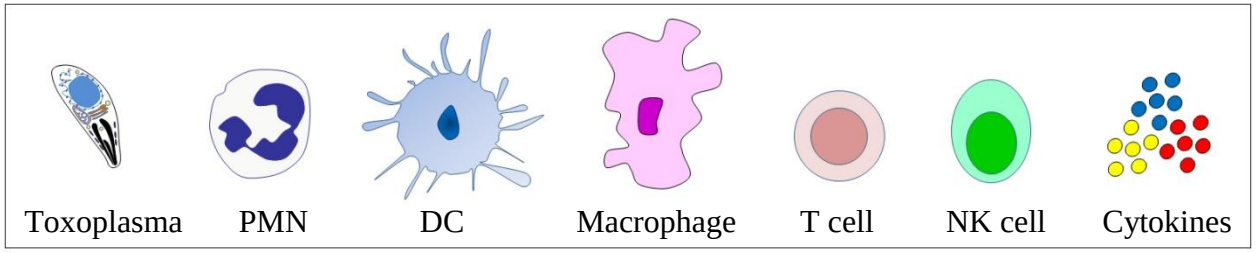
Immunity to *Toxoplasma gondii*

Toxoplasma is a ubiquitous intracellular parasite with three distinct clonal lineages, designated as Types I, II and III (45). Infection with *Toxoplasma gondii* induces a strong and persistent cell-mediated immune response resulting in host protection (46). It is generally believed that once infection is established, the host is resistant to reinfection with the parasite (47). Cells of the innate immune system, most notably macrophages, dendritic cells and neutrophils are responsible for recognition of the parasite and subsequent production of cytokines and chemokines responsible for inducing protective responses in the host, most importantly IL-12 and TNF-α production (48, 49). The production of IL-12 leads to the promotion of T-lymphocyte proliferation into T_h1 IFN-γ producing cells and also has effects on induction of IFN-γ production by (natural killer) NK cells. Both IL-12 and IFN-γ are crucial for the control of the parasite, since depletion (or knock out) of either cytokine or depletion of both cytokines in vivo leads to uncontrolled parasite replication and infected animals die. IL-12 is absolutely critical for the induction of IFN-γ production, since in the absence of IL-12, IFN-γ production is impaired, while the absence of IFN-γ does not entirely abrogate IL-12 production (50). Additional evidence showing that these two cytokines are necessary for the control of *Toxoplasma* comes from studies showing that the administration of IL-12 and IFN-γ promotes survival of infected mice (51, 52).

IFN-γ is generally viewed as the central mediator of host resistance to *T. gondii* (53). This production of IFN-γ activates pathways in innate immune cells that lead to the killing and control of parasites. IFN-γ activated macrophages can become extremely potent cells with high

antimicrobial activity. Activated macrophages undergo nitric oxide (NO) production which leads to the destruction of the parasite. Also, IFN-γ activity on macrophages leads to the production of immunity-related GTPase (IRG) proteins also known as p47 GTPases and pathogen elimination is highly dependent on the IGTP IRG molecule. Killing mechanisms are thought to function by the localization of these proteins to the PV, followed by PV membrane indentation, vesiculation, disruption, stripping of the parasite plasma membrane and eventual fusion/delivery of the parasite to autophagosomes which ultimately fuse with lysosomes killing the parasite (refer to Figure 1.2) (54, 55).

The crucial importance of IFN-γ in the control of parasite replication and host survival is underscored by studies depleting T cells in *Toxoplasma* infected animals. Mice depleted of both $CD4^+$ and $CD8^+$ T cells undergo cyst reactivation and mice die from uncontrolled parasite replication (56). IFN-γ has also been shown to be crucial in the chronic and not just the acute phase of the infection since its continued presence can prevent toxoplasmic encephalitis in infected mice (57). In addition to antibody-mediated depletion of T cells, their importance in the course of a *Toxoplasma* infection is further demonstrated by infecting SCID mice (which lack B and T cells). These mice, although producers of IFN-γ through the activity of NK cells, still succumb to infection highlighting the importance of a continuous presence of $CD4^+$ and $CD8^+$ T cells (58).


Toxoplasma
PMN
DC
Macrophage
T cell
NK cell
Cytokines

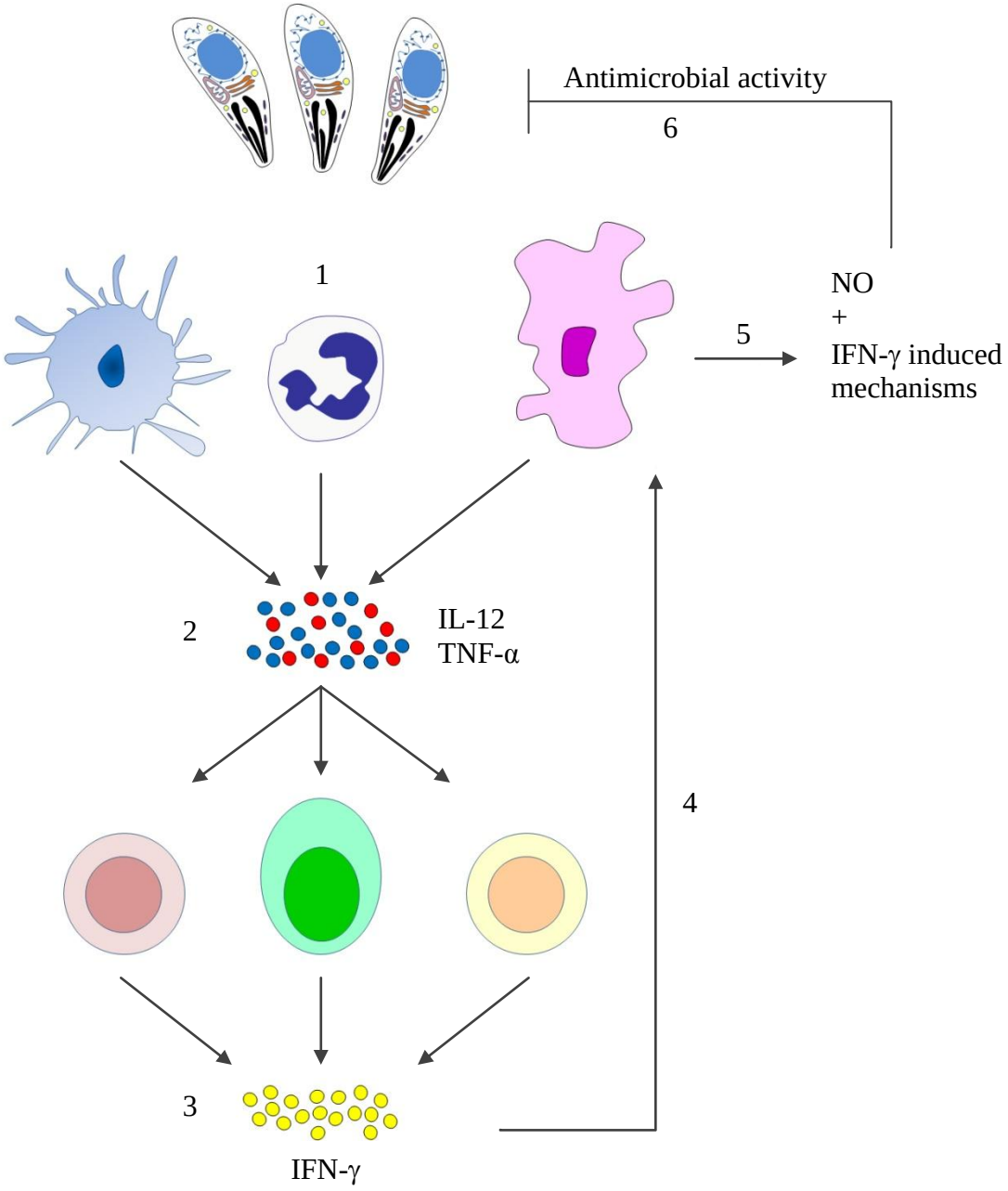

Antimicrobial activity
6
1
5
NO
+
IFN-γ induced
mechanisms
2
IL-12
TNF-α
4
3
IFN-γ

Figure 1.2 (previous page). Immunity to *Toxoplasma gondii*. Cells of the innate immune system, such as DC, PMN and MΦ, are responsible for the recognition of *Toxoplasma* (1). This recognition leads to the production of IL-12 and TNF-α (2) which in turn promotes the production of IFN-γ by NK cells and $CD4^+$ and $CD8^+$ T cells (3). IFN-γ in turn acts on macrophages and activates them (4) to produce NO and other IFN-γ induced killing mechanisms (5) which have toxoplasmacidal effects (6).

Toxoplasma normally causes asymptomatic infection in immunocompetent individuals but the parasite can cause serious clinical disease in immunocompromised hosts (22, 59). Immunity to *T. gondii* consists of a strong T_h1 response that provides protection to the host. However, this inflammatory response can become pathological if not appropriately controlled by down-modulatory cytokines (60, 61). The elucidation of which cells play an important role in mediating immunopathology as well as the different roles down-modulatory cytokines play in contributing to the resolution of *Toxoplasma* induced inflammation have been established using knock-out mice or antibody depletions. Although $CD4^+$ T cells can be extremely beneficial for protection in terms of IFN-γ production, they can also contribute to immunopathology. In a mouse model of ocular toxoplasmosis, $CD4^+$ T cell responses were observed to mediate the ocular inflammatory response to the parasite (62). In another model of infection examining the effect of NK cells in immunopathology, CCR5 was established as being necessary for NK cell homing into infected tissues. Although NK cells produce IFN-γ that is known to have anti-toxoplasmic effects, their lack of recruitment to sites of infection led to a decrease in immune-mediated tissue injury normally seen in WT counterparts, arguing for their role in immunopathology (63).

The host possesses various immunoregulatory and compensatory mechanisms in place that prevent or control *Toxoplasma* induced immunopathology. Studies employing antibody depletions or knock-out mice highlight the importance of IL-10, TGF-β and IL-27 in the control of immune-mediated pathology. IL-10 knock-out mice succumb to infection with *Toxoplasma* fairly quickly with highly elevated serum levels of IL-12, TNF-α and IFN-γ (60). Similarly,

TGF-β-depleted and orally infected mice develop ileitis as a result of lack of gut homeostasis and a rampant ileal inflammatory response that normally would be kept in check by TGF-β producing intraepithelial lymphocytes (64). The role Il-27 plays in downregulating immune responses to *Toxoplasma* was elucidated using IL-27R knock-out mice. When infected, IL-27R KO mice develop lethal inflammatory responses characterized by extensive necrosis in the liver and display elevated levels of IFN-γ, emphasizing the role of IL-27 in the negative control of T cell responses during a *Toxoplasma* infection (65).

Neutrophils

Discovery, differentiation and granulogenesis

Neutrophil granulocytes or polymorphonuclear neutrophilic leukocytes (PMN), are one of the most effective antimicrobial innate immune cells. There are three types of granulocytes: basophils, eosinophils and neutrophils. The neutrophils are the most numerous of all innate immune cells. They were originally described over 100 years ago based on their staining patterns. When subjected to basic hematoxylin and eosin dyes, basophil cytoplasm stains with the basic dye methylene blue, eosinophil cytoplasm is positive for the acid dye eosin red, while neutrophils exhibit neutral cytoplasmic staining, thus the name. Neutrophils are the most abundant leukocyte in the blood, constituting about 50% to 70% of the circulating white blood cells in humans. They have been regarded for years as one of the most important of the induced host innate defenders due to the fact that they are the earliest to arrive at sites of infection or inflammation. Neutrophils are produced in the bone marrow and exit this site once mature. They circulate in the peripheral blood for about 7-10 hours before undergoing apoptosis.

Due to their potentially destructive nature, PMN production by the bone marrow is a tightly controlled process. Granulopoiesis, or differentiation of granulocytes, occurs in the bone marrow over a period of 10 to 14 days and has been divided into stages based on distinctions made on the basis of size, nuclear morphology and granule content. The first recognizable entity is the myeloblast (MB) which originates from a pool of stem cells. This is followed by differentiation into promyelocytes (PM), then myelocytes (MC) followed by metamyelocytes (MM) then band cells (BC) leading to a mature PMN (the segmented neutrophil) (refer to figure 1.3A) (66, 67). During this differentiation process, neutrophils acquire their characteristic granules that allow them to perform many of their functions. Both granulopoiesis and granulogenesis are controlled by a number of transcription factors and disruption of this process can lead to deleterious effects, such as acute myeloid leukemia (AML) (68). Some of the transcription factors involved in differentiation include AML-1, c-myb, C/EBP-α, PU.1, C/EBP-ε and CDP (69).

Neutrophil granules are generated in a sequential fashion that follows the differentiation pathway described above. Intracellular granules of neutrophils belong to one of the following defined categories: azurophil granules (primary granules) which are peroxidase-positive granules, specific granules (secondary granules), gelatinase granules (tertiary granules) both belonging to the peroxidase-negative granules, and secretory vesicles. All these granules share the common characteristics of a phospholipid bilayer membrane and the availability of an intragranular matrix stocked with proteins designed for exocytosis or delivery to the phagosome (70). The azurophil granules are early-appearing and develop in the MB and PM stages of maturation, while specific granules develop after, at the MC and MM stages. Gelatinase granules appear in the BC stage while secretory vesicles are the last to be synthesized and appear when the PMN is in its final

maturation stage. On the other hand, mature PMN discharge granule or vesicle contents following the reverse trend, with the secretory vesicles having the propensity to discharge contents first, while the azurophil granules are the last to discharge contents (71, 72). Secretory vesicles contain the membrane-associated receptors needed in the first stages of a neutrophil immune response, such as the β_2-integrin CD11b/CD18, the complement receptor 1 (CR1), fMLF receptors (formylmethionyl-leucyl-phenylalanine), CD14 (LPS/lipoteichoic acid receptor) and CD16 (FcγIII receptor) (73-75). The major components of the gelatinase granules reflect their role in neutrophil extravasation and diapedesis while the components of the specific granules reflect their role in antimicrobial activities. Both granules share much in common in terms of components and these include lactoferrin, hCAP-18, NGAL, histaminases and lysozyme (76, 77). They also include three major matrix metalloproteases, MMP-8 (collagenase), MMP-9 (gelatinase) and MMP-25 (leukolysin) (78-80). Finally, the azurophil granules are usually the last to be discharged and contain some of the most dangerous of neutrophil proteins. They are stocked with myeloperoxidase (MPO), bacterial permeability increasing protein (BPI), cathepsins, defensins, elastase, lysozyme and proteinase (81-83). All of these granules and their contents allow neutrophils to efficiently combat various microbial agents (refer to Figure 1.3B-E).

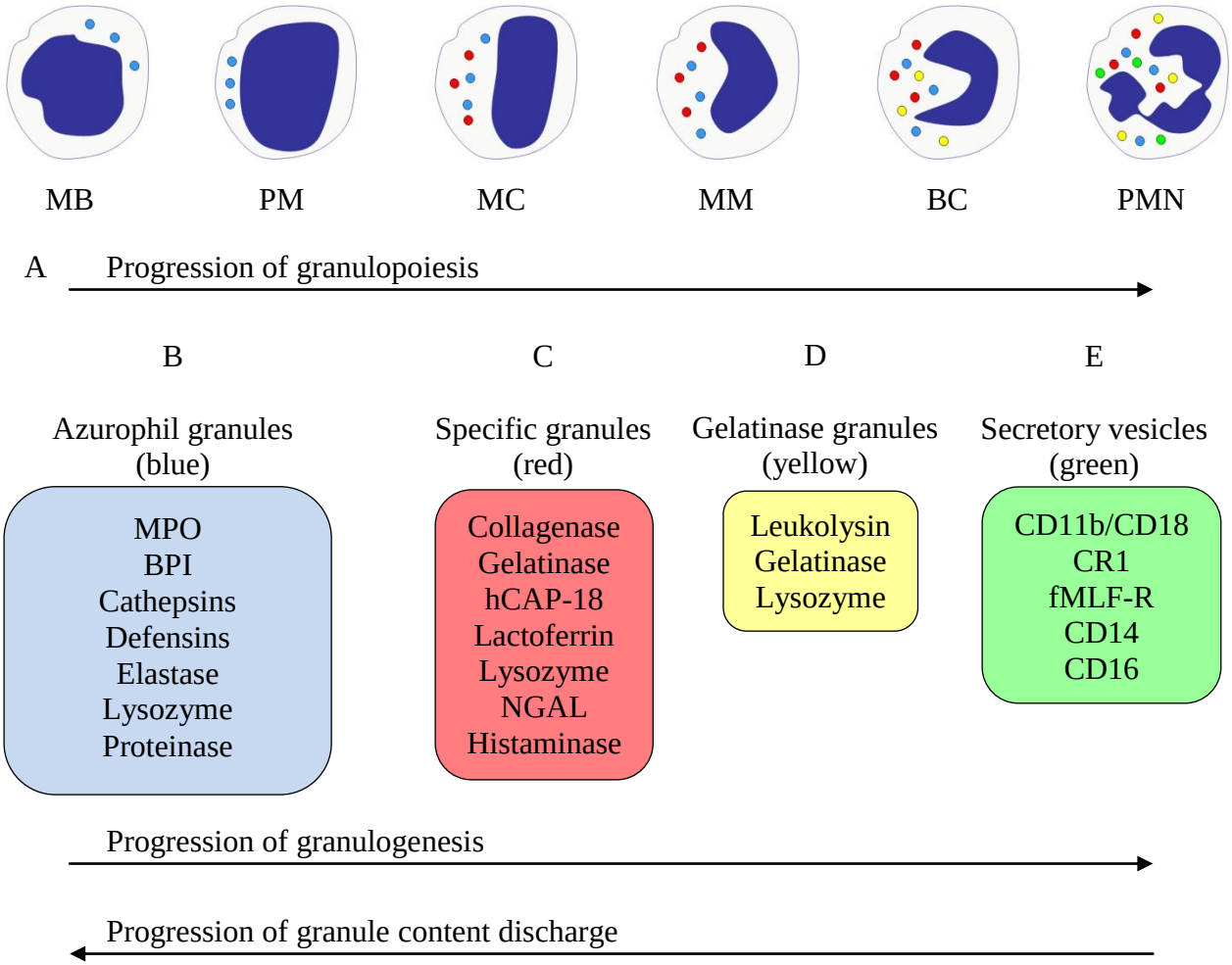


Figure 1.3. Neutrophil granulopoiesis, granulogenesis and granule content. A, Granulopoiesis occurs in the bone marrow and starts with myeloblasts (MB) that differentiate into promyelocytes (PM), then myelocytes (MC) followed by metamyelocytes (MM) then band cells (BC) leading to a mature PMN. B, Azurophil granules develop in the MB and PM stages and contain MPO, BPI, and other proteins and are usually last to discharge their contents. C, Specific granules develop during the MC and MM stages and contain collagenase, gelatinase and various antimicrobial peptides. D, Gelatinase granules develop at the BC stage and contain gelatinase, lysozyme and leukolysins. E, Secretory vesicles are the last to develop and contains mostly surface proteins and receptors and are usually first to discharge their contents.

Neutrophil migration to sites of inflammation

PMN are usually the first cells to arrive at a site of infection and inflammation and this is usually in response to a chemotactic gradient of IL-8 in humans and MCP-2 in mice. Circulating neutrophils undergo a step-by-step migration into infection sites which occurs within minutes to hours of stimulation and involves a series of regulated exocytic events that are essential for the cell's ability to control infection (70). The process is characterized by slow rolling, adhesion strengthening, intraluminal crawling and eventual paracellular and transcellular migration (84). The leukocyte adhesion and migration cascade starts with inflamed vascular endothelial cells expressing P-selectin on their surface. This serves to interact with P-selectin glycoprotein ligand 1 (PSGL-1) expressed on neutrophils and allows these cells to start rolling on the endothelium (85). Also, endothelial cells express E-selectin and ligands for L-selectin which are present on neutrophils (86, 87). The interaction between selectins and their ligands allows for exceptionally strong bonds that can withstand the stress induced by blood flow rates. After the capture and slow rolling step, the neutrophil will release some of its secretory vesicle contents that allow the expression of CD11b/CD18 on the surface of the membrane (88). This expression then allows the engagement of ICAM-1 adhesion molecules on the surface of endothelial cells leading to firm linkage of neutrophils to the endothelial surface and arrest of rolling. At this step, exocytosis of some gelatinase granule components occurs (such as some MMPs) which aid in the degradation of the vascular membrane thus facilitating neutrophil extravasation and transmigration. At this juncture, the neutrophil has now crossed over to the other side of the endothelial cell barrier and will continue its journey to the site of infection or inflammation, releasing on its way contents of specific and azurophil granules that further aid in matrix-degradation and facilitate migration (refer to Figure 1.4A).

Neutrophil intracellular killing mechanisms

Once at the site of infection, neutrophils eliminate invading pathogens through phagocytosis, and the resulting microbe-carrying phagosome fuses with lysosomes where the pathogen can be killed. Neutrophil killing relies on both oxidative and non-oxidative mechanisms. Oxidative mechanisms involve production of reactive oxygen species through the activity of the NADPH oxidase enzyme complex, while non-oxidative mechanisms rely on the release of antimicrobial peptides and proteases (70, 89). The importance of the NADPH oxidase complex in combating diseases and microbial infections is demonstrated in patients with chronic granulomatous disease (CGD), who have a defective oxidative burst, and a profound immunodeficiency in combating microbial and fungal infections (90-92). The crux of this method of killing is the presence of reactive oxygen species (ROS) which are produced as a result of NADPH activity and are eventually transferred into the phagocytic vacuole. This transfer of negatively charged molecules into the phagocytic vacuole results in a charged imbalance that is compensated by the import of K^+ and other positively charged ions into the vacuole. This positive charge flux then mediates the release, solubilization and activation of enzymes, such as proteases, that results in killing (90, 93). One of the most effective antimicrobial defense mechanisms used by neutrophils is the conversion of H_2O_2 into halide products (particularly chloride) by myeloperoxidase (94). The non-oxidative mechanisms of PMN killing involve the deployment of a diverse arsenal of proteins and peptides that are stored in granules described earlier and are directed toward microbial killing and digestion. Microbicidal proteins usually disrupt anionic surfaces of pathogens while proteases will degrade microbial proteins. Together, these killing mechanisms were until recently thought to encompass the whole antimicrobial activity of neutrophils (refer to Figure 1.4B-E).

Neutrophil extracellular traps

A landmark study by Brinkmann et al. identified a previously unrecognized neutrophil anti-microbial mechanism involving the release of nuclear DNA that can play a role in extracellular killing (95). Neutrophil extracellular traps (NETs) are primarily composed of a DNA backbone studded with histones and laced with various anti-microbial peptides that together form an extracellular mesh that can trap and kill microbial pathogens (refer to Figure 1.4F) (96). Histones present in NETs are H1, H2a, H2b, H3 and H4. The protein components have been shown to include bacterial permeability-increasing protein (BPI), myeloperoxidase, cathepsin G, lactoferin, gelatinase, peptidoglycan recognition proteins (PGRPs), calprotectin, as well as neutrophil elastase (95, 97-101). Release of NETs has been described in neutrophils isolated from various species including humans, mice, cows, horses, cats, fish, and even chickens (95, 102-107).

Since their original discovery in neutrophils, formation of extracellular traps has now been described to also occur in eosinophils and mast cells. Key differences in formation and function are that traps from eosinophils occur in live cells, happen fairly quickly (in seconds) and are mitochondrial in source, while release of extracellular traps from mast cells and neutrophils leaves the cells dead, takes longer (minutes to hours), and the DNA is nuclear in origin (108, 109).

Many molecules have been identified as being responsible in triggering NET formation in neutrophils. Some of the most important are: LPS, PMA, GM-CSF/LPS, IL-8, glucose oxidase,

Ca^{2+} ionophore, thapsigargin, TNF, and LPS-activated platelets (95, 110-118). In addition to molecular triggers, neutrophils have been shown to extrude NETs in response to various pathogens and some are susceptible to killing by NETs. These pathogens include *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Shigella flexneri, Salmonella typhimurium, Aspergillus fumigatus, Aspergillus nidulans, Leishmania amazonensis, Leishmania donovani, Plasmodium falciparum, Escherichia coli, Mycobacterium tuberculosis, Listeria monocytogenes* and *Candida albicans* among others (95, 101, 119-127). Of these pathogens, some are killed through a combination of histone and anti-microbial peptide activity. Some of the pathogens susceptible to NET killing include some of the *Streptococcus* and *Staphylococcus* species, *C. albicans, S. typhimurium, E. coli* and *A. nidulans*. There is also evidence that some bacterial pathogens, such as *S. pyogenes* and *S. pneumoniae*, avoid killing by NETs by releasing nucleases (119, 120). For the case of *Leishmania amazonensis*, NET entrapment resulted in parasite killing partly dependent upon presence of histones (126). Other studies with *L. donovani* promastigotes showed formation of NETs, but in this case parasites evaded killing in dependence upon expression of lipophosphoglycan (125). For the case of malaria, there is also evidence for *Plasmodium falciparum*-associated NET formation, and elicitation of anti-nuclear antibodies as a result of DNA release may play a role in pathology in infected children (127). To date, it remains unclear as to whether viruses induce and/or are trapped and inactivated by extracellular traps.

The process of NET formation has been termed NETosis and is believed to be a pathway of programmed cell death that is independent of both apoptosis and necrosis, enabling neutrophils to exert anti-microbial activity even after the cells have died. It is now believed that a series of nuclear and cytoplasmic events must take place before NETosis. These series of events involve

peptidylarginine deiminase (PAD)-mediated histone citrullination, followed by chromatin decondensation, nuclear membrane disintegration and the eventual mixing of both nuclear and cytoplasmic effector proteins before the ultimate step, which is the extrusion of protein-loaded NETs into extracellular space (97, 117, 128, 129). The steps involved in the process of extracellular release and how cytoplasmic membrane integrity is breached remains to be elucidated and molecular mechanisms underlying this novel type of programmed cell death are an active area of investigation (130). NETosis has been shown to depend on the generation of reactive oxygen species and a fully functional NADPH complex (131). Neutrophils isolated from patients with chronic granulomatous disease (CGD), who have defects in NADPH oxidase activity, fail to exhibit NET formation (97, 123). Also, myeloperoxidase and neutrophil elastase have been shown to regulate the formation of NETs (132, 133). Recently, Hakkim et al. identified a signaling pathway involved in extracellular trap formation that involves a Raf-MEK-ERK pathway and that inhibition of this pathway led to inhibition of NET formation (134).

The role of NETs in disease has also been an area of concern and intense study and their role in both infectious and noninfectious diseases is only now becoming more appreciated. For example, during sepsis and upon platelet activation, neutrophils undergoing NETosis can cause endothelial injury and ischemia due to the accumulation of proteases (118). Also, the long-held unexplained observation that patients with preeclampsia have higher numbers of maternal DNA has now been attributed to NETs and their role in entrapping and controlling the release of proinflammatory placenta-derived syncytiotrophoblast microparticles (114). In addition, the possible failure of a host to completely and sufficiently degrade formed extracellular traps has been postulated as the reason behind the development of SLE, usually characterized by the presence of autoantibodies

against DNA and histones (135). Taken together, all these studies contribute to the nascent view that the release of extracellular traps by neutrophils is an indispensable innate immune defense mechanism.

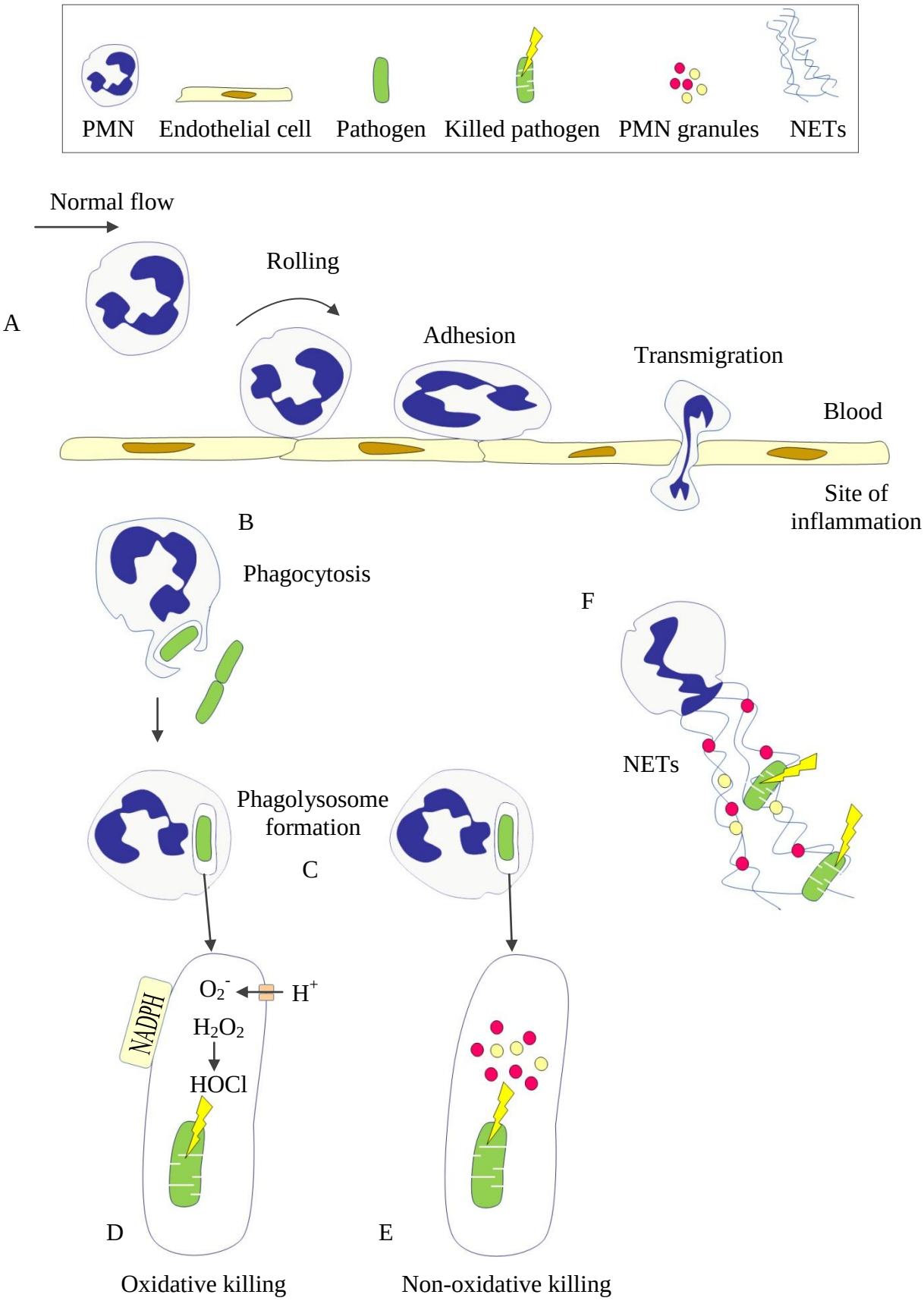

PMN
Endothelial cell
Pathogen
Killed pathogen
PMN granules
NETs
Normal flow
A
Rolling
Adhesion
Transmigration
Blood
Site of inflammation
B
Phagocytosis
F
NETs
Phagolysosome formation
C
O_2^-
H^+
NADPH
H_2O_2
HOCl
D
E
Oxidative killing
Non-oxidative killing

Figure 1.4 (previous page). Neutrophil migration to sites of inflammation and killing mechanisms. Neutrophils migrate to sites of infection or inflammation following a chemotactic gradient. A, Neutrophils circulating in the peripheral blood adhere to the endothelial cells through interactions involving selectin molecules and reduce their flow speed to a slow roll. After these interactions are strengthened, there is strong adhesion followed by transmigration to the tissues. Once at the site of infection, PMN will eliminate pathogens by first phagocytosing them (B) which is followed by the formation of a phagolysosome (C). Once the phagolysosome is formed, pathogens can be killed by oxidative mechanisms (D) or non-oxidative mechanisms (E). F, An extracellular, phagocytosis-independent mechanism for killing involving the production of neutrophil extracellular traps (NETs) is another method of PMN killing. These NETs are granule-content rich.

Neutrophil role in immune modulation

It is becoming increasingly evident that the classical view of neutrophils as short-lived cells with limited ability to affect adaptive immunity is outdated and that their biological significance can extend well beyond this view insofar as they display immunoregulatory activities on other cells of the immune system (136-141). They are capable of producing many chemokines and cytokines that are involved in immune modulation. For example, ample evidence exists showing that these cells have prestored pools of cytokines and chemokines such as IL-4, IL-6, IL-12, TNF-α and MIP-2 (142-146). Of particular interest is the fact that neutrophils can be stimulated to upregulate mRNA expression and release a variety of cytokines and chemokines in addition to those they have prestored thus shaping an immune response (detailed below).

Another novel aspect of neutrophils is their ability to closely interact with T cells and even act as antigen presenting cells (APC) capable of instructing T cells directly, thus shaping the adaptive immune response. There is plenty of evidence in the literature that points to close interactions, whether indirect or direct, between PMN and T cells. Various PMN-derived chemokines and cytokines that are produced at sites of inflammation have the potential to influence T cell activity. For example, chemokines produced by PMN such as CXCL1, CXCL5, CXCL6, CXCL7 and CXCL8 are chemotactic for T cell populations. Cytokines such as IL-4 produced by neutrophils have effects on T_h2 differentiation while IL-12 production has effects on T_h1 differentiation (147). On the other hand, some neutrophil derived products could be T cell inhibitory. Arginase, which is present in copious amounts in azurophil granules, when released to the extracellular milieu can cause a depletion of L-arginine. This depletion of L-arginine at sites

of inflammation has strong effects on T cells and leads to their inhibition since L-arginine is essential for T cell activity (refer to Figure 1.5 A-C) (148).

Direct interactions between neutrophils and T cells, and whether PMN can act as antigen presenting cells (APC) has been an area of intense investigation. Indeed, evidence for this is abundant in humans with chronic inflammatory conditions. Synovial fluid of inflamed joints of patients with rheumatoid arthritis contained neutrophils that expressed MHC class II antigens as well as CD83 and this expression was dependent on activation by T cells (149). Also, neutrophils from patients with Wegener's granulomatosis expressed MHC class II antigens as well as CD80 and CD86, whereas neutrophils from patients with inactive disease did not (150). There is evidence that neutrophils that have undergone MHC class II upregulation can present superantigens (151, 152), and it was shown that mouse neutrophils can stimulate ovalbumin (OVA)-specific T cell proliferation, albeit only with pre-processed antigen (153). However, my recent work shows unequivocally that PMN can act as APC capable of uptake, processing and presentation of OVA antigen to OVA specific T cells. PMN were shown to depend on direct contact with T cells to upregulate MHC class II molecules, CD80 and CD83. As a result, they were able to present antigen to T cells and skew them towards a T_h1 and T_h17 phenotype (refer to Figure 1.5 D) (154).

As a key component of the innate immune system and the inflammatory response, neutrophils make crucial contributions to the recruitment and activation of APC, and especially dendritic cells (DC) (136). Neutrophil-derived cytokines and chemokines can help initiate T_h1 responses

during a *Toxoplasma* infection by influencing DC function. *Toxoplasma* triggered neutrophil synthesis of CCL3, CCL4, CCL5 and CCL20 and these molecules were highly chemotactic for immature DC (155). Also TNF-α production by neutrophils is responsible for the upregulation of CD40 and CD86 expression on DC and supernatants from *Toxoplasma* stimulated PMN can induce DC production of IL-12 and TNF-α (156). DC and neutrophils can also interact directly with each other through cell-to-cell contact between the DC-specific C-type lectin DC-SIGN and the β_2-integrin Mac-1, as well as CEACAM1 on neutrophils, and it was also shown that TNF-α production by neutrophils is essential for inducing DC maturation, evidenced by upregulation of CD83 on these cells (157, 158). These data taken collectively provide a novel cellular link between innate and adaptive immunity and enables neutrophils to modulate T cell responses through their interactions with dendritic cells (refer to Figure 1.6) (139).

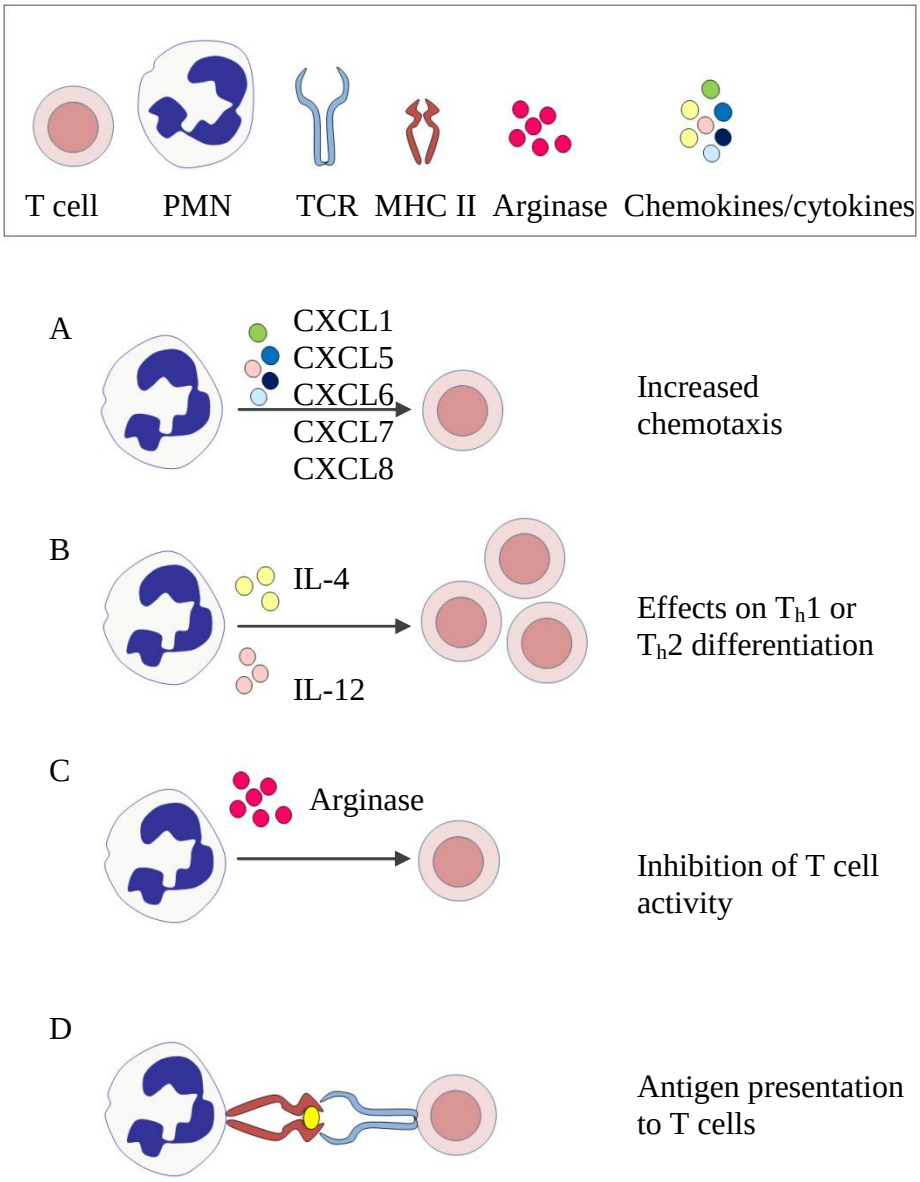
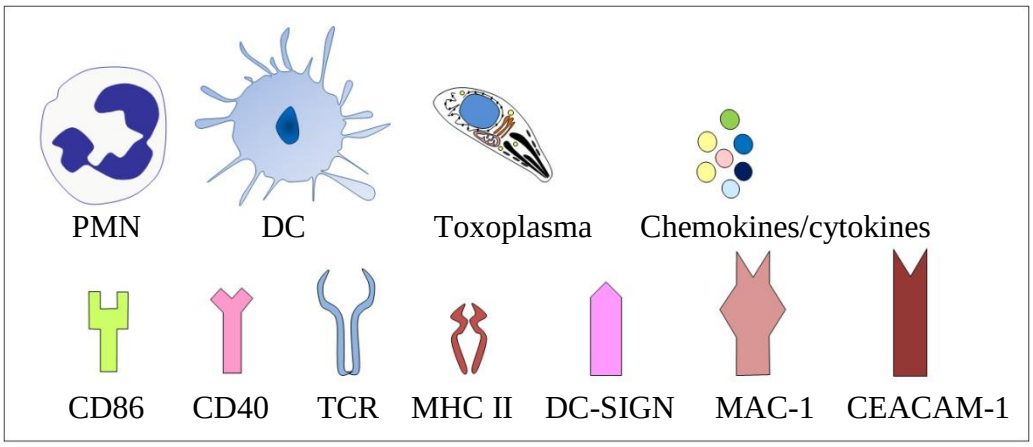


Figure 1.5. PMN shaping of T cell responses. A, PMN derived CXCL1, CXCL5, CXCL6, CXCL7 and CXCL8 are highly chemotactic for T cells. B, IL-4 and IL-12 cytokines produced by neutrophils can have effects on T_h1 and T_h2 differentiation. C, Arginase contained in PMN azurophil granules and released to the extracellular milieu can deplete extracellular L-arginine required for T cell activity thus inhibiting it. D, PMN acquire APC characteristics through contact with T cells, upregulation MHC class II and costimulatory molecules, thus processing and presenting antigen to T cells.


PMN
DC
Toxoplasma
Chemokines/cytokines
CD86
CD40
TCR
MHC II
DC-SIGN
MAC-1
CEACAM-1

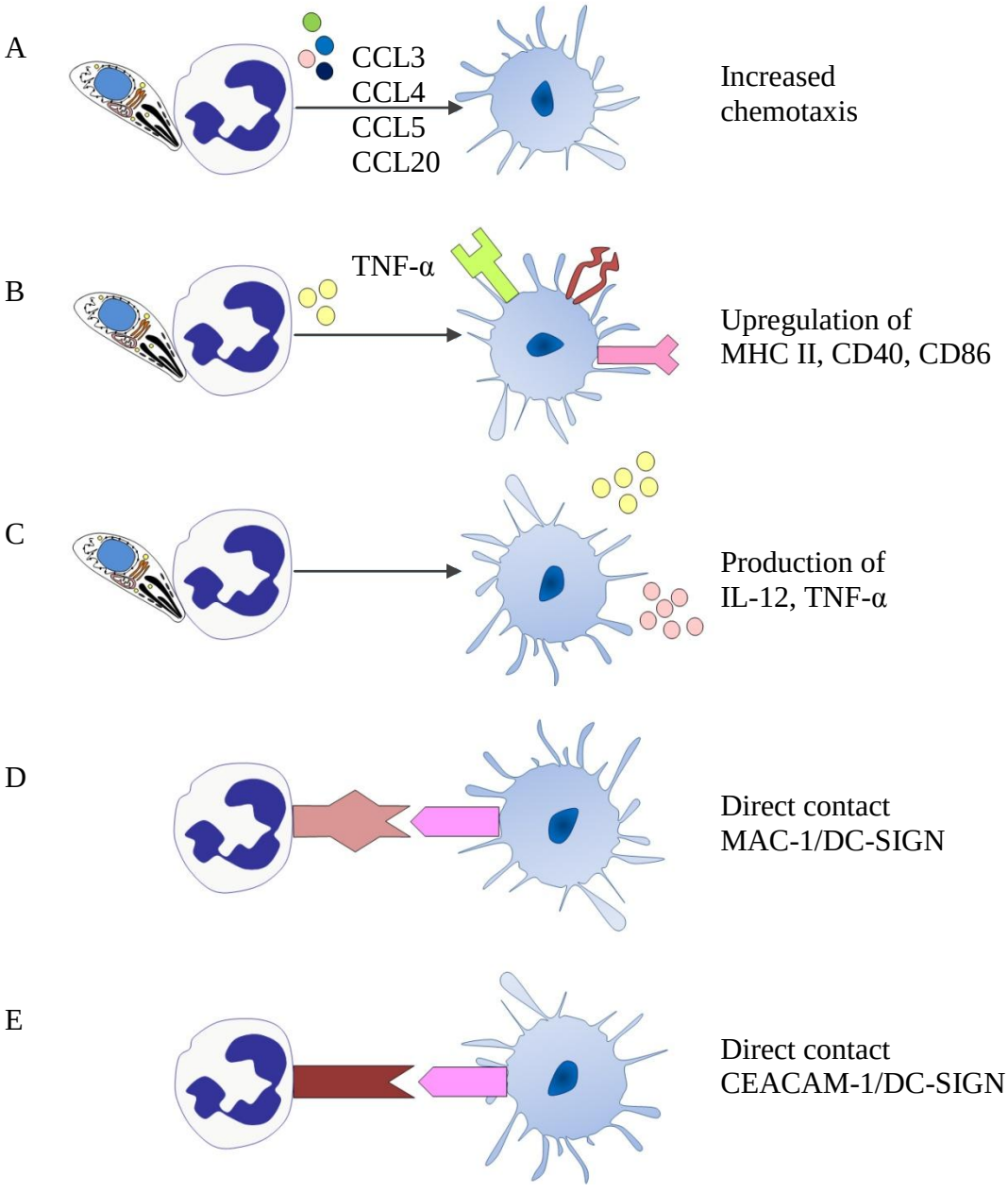

A
CCL3
CCL4
CCL5
CCL20
Increased chemotaxis
B
TNF-α
Upregulation of MHC II, CD40, CD86
C
Production of IL-12, TNF-α
D
Direct contact MAC-1/DC-SIGN
E
Direct contact CEACAM-1/DC-SIGN

Figure 1.6 (previous page). PMN immunomodulatory role through interactions with DC. A, *Toxoplasma* stimulated PMN release of CCL3, CCL4, CCL5, and CCL20 increasing chemotaxis of immature DC. B, TNF-α production by PMN after *Toxoplasma* stimulation leads to upregulation of MHC II, CD40 and CD86 on immature DC. C, Supernatants from *Toxoplasma* stimulated PMN induce production of IL-12 and TNF-α by DC. D, Direct contact between MAC-1 on PMN and DC-SIGN on DC. E, Direct contact between CEACAM-1 on PMN and DC-SIGN on DC.

Importance of neutrophils during infection-mouse studies

The role of neutrophils in microbial infections has been an area of intensive research. Mice rendered neutropenic are extremely susceptible to a variety of infectious agents. Mice depleted of neutrophils, using the anti-Gr-1 antibody (which recognizes the abundantly expressed neutrophil surface marker Ly6C/G), fail to control infections with *Aspergillus fumigates, Candida albicans, Chlamydia trochomatis, Legionella pneumophilia, Leishmania infantum, Leishmania major, Toxoplasma gondii* and *Listeria monocytogenes* among other pathogens (159-173). While the effect of neutrophils in some infections is clearly protective and necessary for survival, neutrophils can contribute to disease in other infections. Mice depleted of Gr-1^+ cells and infected with *Plasmodium berghei* ANKA survive longer than mice whose neutrophils were not depleted (174). Also, in a mouse model of *Cryptococcus neoformans* infection, pulmonary infection of Gr-1^+ depleted mice resulted in better survival rates when compared to non-depleted and infected mice (175).

Of interest to our laboratory is the role neutrophils play in the context of a *Toxoplasma* infection. There is evidence that neutrophils are important in a *Toxoplasma* infection, inasmuch as they are rapidly recruited to the site of infection, lack of recruitment in mice lacking CXCR2, the major receptor for the neutrophil chemotactic IL-8 like chemokines, correlates with increased susceptibility. This CXCR2 deficiency resulted in the establishment of higher cyst numbers in the brains of mice when compared to WT controls as well as lower levels of IFN-γ (176). Our laboratory has shown that neutrophils produce a variety of cytokines and chemokines in response to a *Toxoplasma* infection. Of particular importance is the production of IL-12, TNF-α, MIP-1β, MIP1-α in human neutrophils, and MyD88-dependent IL-12 and MCP-1 in mouse neutrophils in

response to parasite stimulation (49, 177). Also of interest is the fact that during a *Toxoplasma* infection, neutrophils with prestored IL-12 are recruited to the site of infection (142). Our laboratory has previously shown that mice depleted of Gr-1$^+$ cells and infected with *T. gondii* succumb to the parasite within ten days postinfection with a highly dysregulated T_h1 cytokine profile. This dependency of Gr-1$^+$ cells was shown to be crucial in the early phases of infection, since mice depleted of cells starting at 6 days postinfection survive acute infection. This depletion also led to uncontrolled tachyzoite replication with exacerbated lesions in multiple organs (178). The importance of neutrophils in acute infection and the role these cells play in the first days of infection has also been explored by other groups using different pathogens. In addition to *Toxoplasma,* infection models using *Mycobacterium tuberculosis, Leishmania infantum* and *Candida albicans* have highlighted the importance of neutrophils during early stages of infection, since the depletion of neutrophils in the chronic phases of infections had no bearing on the outcome of disease (161, 165, 179).

Historically, mice were rendered neutropenic through the administration of the anti-Gr-1 depleting antibody which recognizes the abundantly expressed Ly6C/G neutrophil surface molecule. While this antibody depletes neutrophils very efficiently, it has recently been shown that other cells of the immune system can express the Ly6C/G surface molecule. The Gr-1 surface marker can also be found on a recently described inflammatory monocyte population of cells, as well as on plasmacytoid dendritic cells leading to mixed interpretations of anti-Gr-1 antibody depletions (180-182). However, another characteristic hallmark of neutrophils is their surface expression of Ly6G molecules (182). A more recent study makes use of a recently available monoclonal antibody that targets the neutrophil-specific Ly6G surface molecule (183).

This antibody has not been shown to react with Gr-1^{+} cells and will not deplete non-neutrophil specific cell populations. As such, the role of neutrophils in vivo can be assayed by the use of Ly6G-specific monoclonal antibody to deplete these cells. Data presented in chapter 4 uses this anti-Ly6G monoclonal antibody to evaluate the role of neutrophils in a *Toxoplasma* infection.

It is becoming increasingly clear that neutrophils are not just scavenging cells but that they can also initiate and influence the adaptive immune response during the control of intracellular pathogens (137). Although neutrophils make small amounts of cytokines and effector molecules on a per-cell basis, their sheer number likely makes them important decision-making immunoregulatory cells that are crucial in mounting effective immune responses (136). Taking all these data collectively, neutrophils produce a variety of cytokines and chemokines that can alter or make various contributions to an immune response.

Brief outline of dissertation research

Neutrophils have long been regarded as one of the most important cells of the innate immune system. The long-held view has been that they are "kamikaze" type cells that migrate to a site of inflammation or infection, perform anti-microbial functions, and then die through apoptosis. Also, it has long been postulated that their killing mechanisms are mostly intracellular and that they lacked any extracellular killing mechanisms or that their role ends at death. PMN ability to modulate an immune response well beyond innate immunity has always been an area of speculation.

This thesis examines newly emerging roles for neutrophils. **Chapter 2** shows clear evidence that neutrophils can act as professional APC capable of processing and presenting antigen to T cells. Neutrophils express T cell contact dependent MHC class II and costimulatory molecules necessary for antigen presentation. They also instruct T cells to differentiate into T_h1 and T_h17 cells independently of exogenous cytokine addition. **Chapter 3** describes neutrophil extracellular traps and their role in the control of *Toxoplasma*. Data presented in Chapter 3 shows that *Toxoplasma* can induce NET formation in both mouse and human neutrophils in an ERK pathway dependent fashion. Data presented in this chapter also shows that NET formation by neutrophils in response to *Toxoplasma* can play a role in the control of the tachyzoite. **Chapter 4** addresses the role of neutrophils in a *Toxoplasma* infection by making use of the neutrophil-specific anti-Ly6G antibody to deplete mice in vivo. This neutrophil depletion led to a dysregulated pulmonary immune response characterized by higher levels of cytokines and dysregulated DC function along with overactive T cells. **Chapter 5** summarizes the data and results from Chapters 2, 3 and 4 and discusses the relevance of these finding and their contribution to the advancement of both the neutrophil and *Toxoplasma* fields as well as discusses future directions for each project.

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CHAPTER 2

Mouse Neutrophils are Professional Antigen-Presenting Cells Programmed to Instruct Th1 and Th17 T Cell Differentiation*

* Reprinted from Delbert S. Abi Abdallah, Charlotte E. Egan, Barbara A. Butcher, and Eric Y. Denkers. Mouse neutrophils are professional antigen-presenting cells programmed to instruct Th1 and Th17 T-cell differentiation. *Int. Immunol.* (2011) 23 (5): 317-326. *Copyright 2011. The Japanese Society for Immunology.*

Abstract

Neutrophils play a major role in the innate immune system and are normally considered to be short-lived effector cells that exert anti-microbial activity and sometimes immunopathology. Here, we show that these cells possess an additional function as professional antigen-presenting cells capable of priming a T_h1- and T_h17-acquired immune response. Using flow cytometry, fluorescence microscopy and western blotting, we show that mouse neutrophils express MHC class II and co-stimulatory molecules CD80 and CD86 after T-cell co-incubation. Neutrophils pulsed with ovalbumin (OVA) process and present peptide antigen to OVA-specific T cells in an MHC class II-dependent manner. Importantly, we demonstrate that neutrophils can prime antigen-specific T_h1 and T_h17 immune responses even without the addition of exogenous cytokines to cell cultures.

Introduction

Neutrophils are well known to rapidly and in large number accumulate at sites of infection and inflammation. The classical view of these cells is that they function as kamikazi pilots, following chemokine gradients to target sites where they kill microbes, simultaneously causing collateral tissue damage through discharge of toxic mediators that also result in their own death. Subsequent phagocytosis of apoptotic neutrophils is thought to allow their immunologically silent removal. Thus, neutrophils are often dismissed as short-term effectors that have no lasting impact on the ensuing adaptive immune response to infection (1).

However, there is mounting evidence that polymorphonuclear leukocytes (PMN) may play more complex and nuanced roles in immunoregulation. Although the lifespan of neutrophils in the

blood is only 8–12 hr, proinflammatory mediators prevent onset of apoptosis, increasing their longevity greatly (2). It is also clear that neutrophils produce a large number of cytokines and chemokines during infection (3, 4). While on a per cell basis, the amount of any given neutrophil-derived cytokine or chemokine is often less than that produced by dendritic cells (DC) or macrophages, the sheer number of PMN (they are the most common leukocyte) suggests that on a population basis, neutrophils can contribute significantly to the cytokine response during infection. There is evidence that neutrophils differentiate into distinct subsets based upon Toll-like receptor expression and reciprocal secretion of IL-12 and IL-10 (5, 6). The ability of neutrophils to produce immunoregulatory cytokines and chemoattractants has led some investigators, including us, to propose that these cells play a role in recruitment and activation of DC during infection (7, 8).

Another immunoregulatory aspect of neutrophil function comes from evidence that these cells may be induced to express MHC class II molecules (9). While PMN from healthy donors do not express MHC class II glycoproteins, cytokines such as IFN-γ, granulocyte-monocyte colony-stimulatory factor (GM-CSF), and IL-3 can stimulate up-regulation (10-12). Interestingly, at least two of these mediators (IFN-γ and GM-CSF) also increase neutrophil survival, suggesting that prolonged contact between neutrophils and T cells is possible (13-15). It has also been reported that neutrophils can express co-stimulatory molecules CD80 and CD86, as well as the DC marker CD83. This has led some to propose that PMN are able to transdifferentiate into DC-like cells under appropriate stimulatory conditions (16-18). There is evidence that human neutrophils mediate proliferation of T cells in response to superantigen (11, 19), and it was recently shown that mouse neutrophils can stimulate ovalbumin (OVA)-specific T cell

proliferation (20). However, whether these responses truly reflect an ability to process and present antigen has been less clear.

Here, we show for the first time unequivocal evidence that neutrophils function as bona fide professional antigen-presenting cells (APCs), inducibly expressing MHC class II and co-stimulatory molecules and stimulating MHC class II-dependent proliferation of OVA-specific T cells after pulsing with antigenic peptide or intact OVA. Most importantly, we show that antigen-pulsed neutrophils are potent inducers of T_h1 and T_h17 differentiation *in vitro* independent of any exogenous cytokine addition. Together, our results demonstrate that neutrophils function as a new type of professional APC specializing in driving generation of proinflammatory T cell effectors.

Methods and materials

Mice

Female C57BL/6 and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) or Taconic Farms (Germantown, NY, USA) and used at 6–8 weeks of age. MHC class II knockout mice on a C57BL/6 background (B6.129-*H2-Ab1*^{tm1Gru} N12) were purchased from Taconic Farms. OT-II transgenic mice expressing an OVA-specific T cell receptor on a *Rag-1*$^{-/-}$C57BL/6 background [C57BL/6-*Rag2*^{tm1Fwa} Tg(TcraTcrb)425Cbn] were obtained from Taconic Farms. All mice were maintained in the Transgenic Mouse Core Facility at the Cornell University College of Veterinary Medicine, which is accredited by the American Association for Accreditation of Laboratory Animal Care.

Percoll gradient purification of neutrophils

Mice were intraperitoneally (i.p.) injected with 0.5 ml of 10% thioglycollate (Becton Dickinson, Franklin Lakes, NJ, USA) and 18–20 hr later, peritoneal exudate cells (PECs) were collected by washing the peritoneal cavity with ice-cold PBS (Cellgro, Manassas, VA, USA). After washing cells twice with PBS, neutrophils were purified by continuous Percoll gradient centrifugation as described elsewhere (21). Briefly, Percoll (GE Healthcare, Fairfield, CA, USA), adjusted to pH 7.4, was mixed at a ratio of 1: 9 with PEC re-suspended in PBS. The mixture was then transferred to a 10 ml polycarbonate centrifuge bottle and ultracentrifugation was performed at 60 000 × *g* for 65 min at 4°C using a 50 Ti rotor (Beckman Centrifuges, Brea, CA, USA). The layer enriched for neutrophils was collected using a gel-loading pipette tip. Neutrophils were subsequently washed twice with PBS and re-suspended in complete DMEM (cDMEM), consisting of 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 30 mM HEPES (all purchased from Invitrogen Life Technologies, Carlsbad, CA, USA), 10% bovine growth serum (Hyclone, Logan, UT, USA) and 0.05 mM β-mercaptoethanol in DMEM. Neutrophil preparations were consistently >95% pure, as determined by flow cytometry and differential staining.

Bone marrow-derived DC

Bone marrow derived DC were prepared from flushed femur bone marrow cells. Briefly, bone marrow cells were suspended in RPMI (Cellgro, Manassas, VA, USA) supplemented with 10% FCS (Hyclone), 100 U ml^{-1} penicillin (Gibco, Carlsbad, CA, USA), 100 μg ml^{-1} streptomycin (Gibco) as well as GM-CSF (20 ng ml^{-1}; Peprotech, Rocky Hill, NJ, USA). Cells were fed 3 and

6 days after culture initiation with fresh medium and cells were collected on day 9 for use in experiments.

Magnetic cell sorting of CD4 T cells

Splenocyte single-cell suspensions were obtained by gently mashing spleens and passing them through a 70-μm filter (BD Falcon, Franklin Lakes, NJ, USA) followed by erythrocyte lysis with red blood cell lysis buffer (Sigma-Aldrich, St Louis, MO, USA). Cells were centrifuged at 300 × *g* for 10 min at 4°C. The pellet was re-suspended in MACS buffer (PBS, 0.5% BSA and 2 nM EDTA) and MACS anti-CD4 magnetic beads (Miltenyi Biotec, Auburn, CA, USA) were added. The sample was mixed and incubated for 15 min at 4°C. Cells were washed using MACS buffer and centrifuged at 300 × *g* for 10 min. The cell pellet was re-suspended in MACS buffer and CD4-positive and -negative fractions were separated using an AutoMACS Separator.

Flow cytometry

Single-cell suspensions were incubated for 30 min at 4°C with FACS buffer (PBS, 1% bovine growth serum and 0.01% NaN_3) containing 10% normal mouse serum to block Fc receptor binding. Samples were centrifuged and pellets were re-suspended in FACS buffer containing fluorochrome-conjugated antibody for 30 min at 4°C. The antibodies used in this study were anti-Gr-1 FITC (BD Biosciences, San Jose, CA, USA); PE-conjugated anti-Ly6G (BD Biosciences), PE-conjugated anti-CD80, PE-conjugated anti-CD86 (BD Biosciences), PE-conjugated anti-MHC class II and anti-CD4 conjugated to allophycocyanin. Antibodies were purchased from eBioscience (San Diego, CA, USA) unless indicated otherwise. After washing,

cells were re-suspended in FACS buffer and collected on a BD FACSCalibur flow cytometer. Data analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA).

Intracellular cytokine staining was performed in neutrophil T-cell cultures following a previously described protocol (22). At day 3, after initiating cultures of OT-II T cells and OVA peptide-pulsed neutrophils, cells were pelleted and new medium supplemented with IL-2 (10 ng ml^{-1}; Peprotech) was added. At day 6, cells were collected, washed and stimulated with phorbol myristate acetate (10 ng ml^{-1}; EMD, Darmstadt, Germany) and ionomycin (1 μg ml^{-1}; Calbiochem, Darmstadt, Germany) for 6 hr in the presence of Brefeldin A. Cells were subsequently permeabilized and stained for intracellular cytokines and surface stained for membrane antigens.

Cell sorting

In some experiments, high purity neutrophils were obtained by staining thioglycollate-elicited cells with PE-conjugated antibody specific for Ly6G (1A8; BD Pharmingen, San Diego, CA, USA) for 30 min at 4°C. After extensive washing, cells were re-suspended in PBS with 1% bovine growth serum (Hyclone) and sorted based upon expression of Ly6G using a FACSAria cell sorter (BD Biosciences). In other experiments, $CD4^+$ T cells were isolated by flow cytometric sorting after staining cells with anti-CD4 antibody conjugated to allophycocyanin. The purity of sorted populations was routinely >98%.

5-(and 6-)Carboxyfluorescein diacetate succinimidyl ester staining of CD4 T cells

CD4-positive T cells were stained with 5-(and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Carlsbad, CA, USA) according to the manufacturer's instructions.

Briefly, cells were re-suspended in PBS with 0.1% BSA and CFSE stock solution was used to yield a final concentration of 10 μM. Cells were incubated at 37°C for 10 min. The staining was quenched by adding five volumes of ice-cold cDMEM followed by incubation on ice for 5 min. CFSE-stained cells were further washed for a total of three washes using cDMEM.

Neutrophil and CD4 T cell co-cultures

Neutrophils, purified by Percoll gradient centrifugation or flow sorting, were either pre-incubated with medium alone, OVA peptide (amino acids 323–339; AnaSpec, Fremont, CA, USA) at a concentration of 2 μg ml^{-1} or whole OVA antigen (Thermo Scientific, Rockford, IL, USA) at a concentration of 20 μg ml^{-1} for 4 h at 37°C. After extensive washing in cDMEM, immunomagnetic bead-purified, or in some cases flow sorted, CFSE-labeled OT-II $CD4^+$ T cells were added to the neutrophils. After 4 days of culture, cells were collected and surface stained for CD4 as described above and CFSE peak dilutions were assayed using flow cytometry. Anti-MHC class II antibody blocking experiments were performed using Ab M5/114 (eBioscience) at a concentration of 5 μg ml^{-1}. Transwell contact inhibition experiments were performed using a 24-well transwell plate (Costar, Lowell, MA, USA). Neutrophils and T cells were co-incubated in direct contact or separated by the transwell membrane with neutrophils in the lower chamber and T cells in the upper chamber. After incubation for 2 hr at 37°C, neutrophils were collected and stained for MHC class II expression as described above.

Western blotting

Percoll gradient purified neutrophils, isolated by flow cytometric sorting of $Ly6G^{high}$ cells after incubation with T cells, were lysed using SDS sample reducing buffer and stored at -80°C.

Samples were subsequently resolved by SDS-PAGE, and immunoblotted using an anti-MHC class II Aβ-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or stripped and re-probed for anti-GAPDH-specific antibody (Cell Signaling Technology, Danvers, MA, USA) as a loading control. Blots were visualized using an ECL-based detection system (Thermo Scientific).

Cytokine ELISA

IFN-γ in culture supernatants was measured using a commercial kit according to the manufacturer's recommendations (eBioscience). IL-17 was measured using a kit from R&D systems and IL-4 was measured using a kit from BD Biosciences (BDoptEIA).

Immunofluorescence microscopy

Neutrophils before or after T cell co-incubation were centrifuged on to glass cover slips for 5 min at 750 r.p.m. Cover slips were fixed with 3% paraformaldehyde (20 min, room temperature), then blocked with normal mouse serum and FCS-supplemented PBS for an hour at room temperature. Cover slips were incubated with anti-Gr-1 FITC (BD Biosciences); and PE-conjugated anti-MHC class II (eBioscience) for an hour at room temperature. After washing in PBS, cover slips were mounted with ProLong Antifade containing 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Images were collected with an Olympus BX51 fluorescence microscope equipped with a DP 70 camera using Olympus DP controller software and Olympus DP manager software.

Statistical analyses

Statistical analyses were performed using the Prism Software. Unpaired *t*-tests were performed to analyze data. *P* values <0.05 were considered significant.

Results

Isolation of mouse neutrophils

To obtain neutrophils, mice were i.p. injected with 10% thioglycollate and PEC were collected 18-22 hr later. The cell population obtained was routinely 60-80% neutrophils with macrophages, mast cells, eosinophils and lymphocytes making up the remaining fraction of cells (data not shown). To purify neutrophils, PEC were centrifuged over a Percoll continuous density gradient consistently yielding a population of cells that were 96-98% positive for the neutrophil-associated marker Ly6G (Figure 2.1A). There was routinely <0.5% $CD11c^+$, $F4/80^+$, and $CD19^+$cells in this population (Figure 2.1D-F). The $Ly6G^+$ population could be divided into $Ly6G^{high}$ and $Ly6G^{interm}$ cells (94.7 and 1.7%, respectively; Figure 2.1A). These two populations were isolated by flow cytometric sorting of $Ly6G^{high}$ (Figure 2.1B) and $Ly6G^{interm}$ (Figure 2.1C) cells. Differential staining of these cells showed that essentially all were neutrophils, as determined by their characteristic multilobed nuclei (Figure 2.1B and C, insets). We speculate that the $Ly6G^{interm}$ PMN are recent bone marrow emigrants recruited in response to thioglycollate injection because it is known that expression of this marker increases with neutrophil maturation (23).

Expression of MHC and costimulatory molecules on mouse neutrophils

We previously reported that mouse neutrophils express the molecule CD80 that is associated with co-stimulation of T lymphocytes during MHC-restricted antigen presentation (Figure 2.2F, inset) and (21). Therefore, we asked whether thioglycollate-elicited PMN also express CD86 and MHC class II molecules. We found that freshly isolated cells expressed neither MHC class II molecules (Figure 2.2A and B) nor did they express CD86 (Figure 2.2E, gray histogram). However, after 2 hr co-incubation with purified $CD4^+$ T cells, we detected up-regulation of MHC class II molecules (Figure 2.2C and D) as well as increased expression of CD86 (Figure 2.2E) on PMN. Levels of CD80 on neutrophils were also slightly up-regulated following T cell co-incubation (Figure 2.2F).

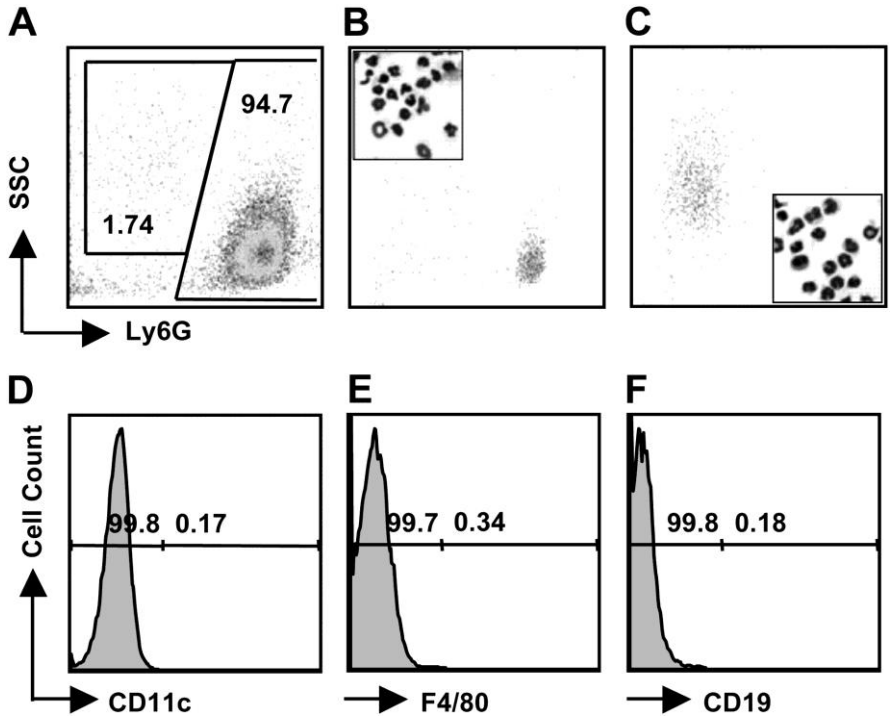


Figure 2.1. Source of neutrophils and absence of APC populations in purified neutrophils. (A) Expression of Ly6G by thioglycollate-elicited neutrophils isolated by centrifugation over Percoll. The numbers indicate the relative percentage of cells falling within each indicated gate. PMN were sorted into Ly6G high (B) and intermediate (C) expressing populations. Insets in panels B and C show the morphological appearance of the flow-sorted populations. There was minimal cross contamination as determined by staining for DC (CD11c, panel D), macrophages (F4/80, panel E) and B cells (CD19, panel F). Data are representative of at least three independent experiments.

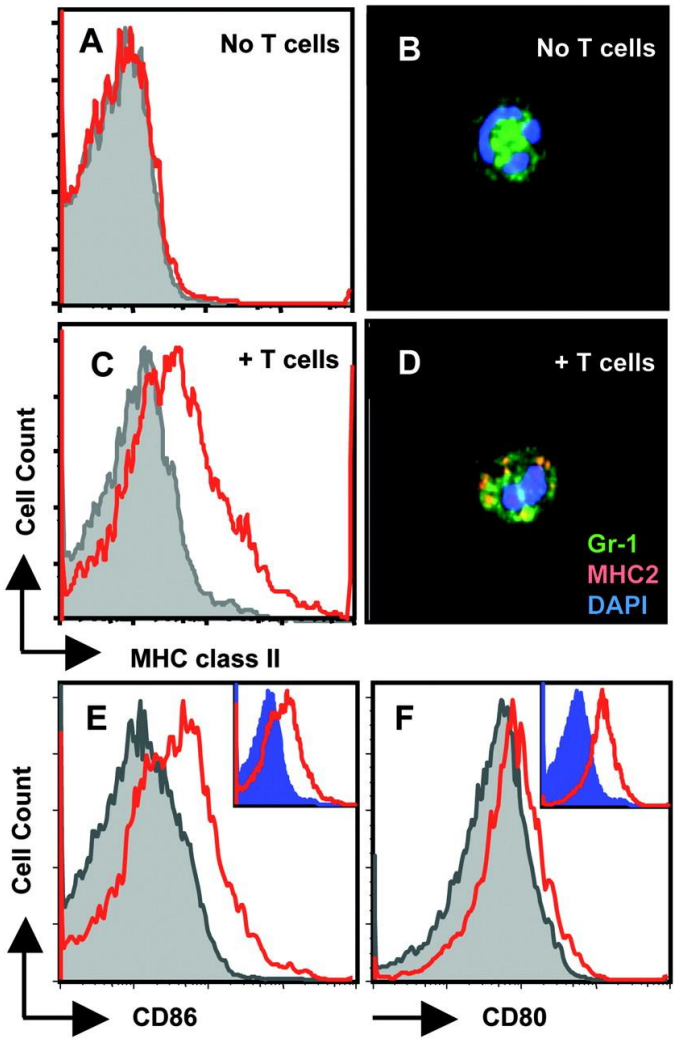

A
No T cells
B
No T cells
C
+ T cells
D
+ T cells
Gr-1
MHC2
DAPI
Cell Count
MHC class II
E
F
Cell Count
CD86
CD80

Figure 2.2 (previous page). Co-incubation with T cells induces expression of MHC class II and co-stimulatory molecules on neutrophils. (A and C) Expression of MHC class II molecules on Ly6C/G (Gr-1)-gated neutrophils without (A) or with (C) 2 hr T cell co-incubation (red histograms). Gray shaded histograms show isotype antibody staining. (B and D) Neutrophils were imaged by fluorescence microscopy after staining with anti-Ly6C/G antibody (green), anti-MHC class II antibody (red). Nuclei were stained with DAPI (blue). MHC class II expression on neutrophils without (B) and with (D) T cell co-incubation is shown. (E and F) Expression of CD86 (E) and CD80 (F) on Ly6C/G-gated neutrophils without T cell co-incubation (shaded histogram) relative to expression after T cell co-incubation (red histogram). The insets in panel E and F show isotype antibody staining (shaded blue histogram) compared with CD86 and CD80 expression on neutrophils with T cell co-incubation respectively (red histogram). MHC class II and co-stimulatory molecule flow cytometry data are representative of at least four independent experiments. The microscopy data are representative of two independent experiments.

To further substantiate expression of MHC class II molecules on PMN, we co-incubated neutrophils with magnetic bead purified $CD4^+$ T cells (Figure 2.3A), then we re-isolated neutrophils 2 hr later by cell sorting following labeling with Ly6G-specific antibody (Figure 2.3B). Neutrophil cell lysates were subjected to western blot analysis using an MHC class II Aβ-specific antibody. As shown in Figure 2.3(C), neutrophils lysed without pre-incubation with T cells (Contr) expressed minimal levels of Aβ chain, consistent with data shown in Figure 2.2(A and B). However, supporting the flow cytometry results in Figure 2.2, neutrophil lysates prepared after co-incubation with T cells (Post-T) clearly contained increased levels of the MHC class II Aβ molecule (Figure 2.3C). This difference in expression is not due to unequal loading of samples as shown by blotting against GAPDH (Figure 2.3C).

We sought to determine whether T cell-driven expression of MHC class II on PMN was dependent upon release of soluble factors or direct cell-to-cell contact. To answer this question, neutrophils and T cells were incubated together or separated by a Transwell membrane. Freshly isolated neutrophils incubated alone did not express MHC class II molecules (Figure 2.4A). Neutrophils incubated in direct contact with T cells up-regulated expression of MHC class II molecules (Figure 2.4B). Strikingly, up-regulation of MHC class II molecules on neutrophils was completely abrogated when the cells were separated by a Transwell membrane (Figure 2.4C). We conclude that cell contact is required for T lymphocyte-triggered up-regulation of MHC class II molecules on PMN.

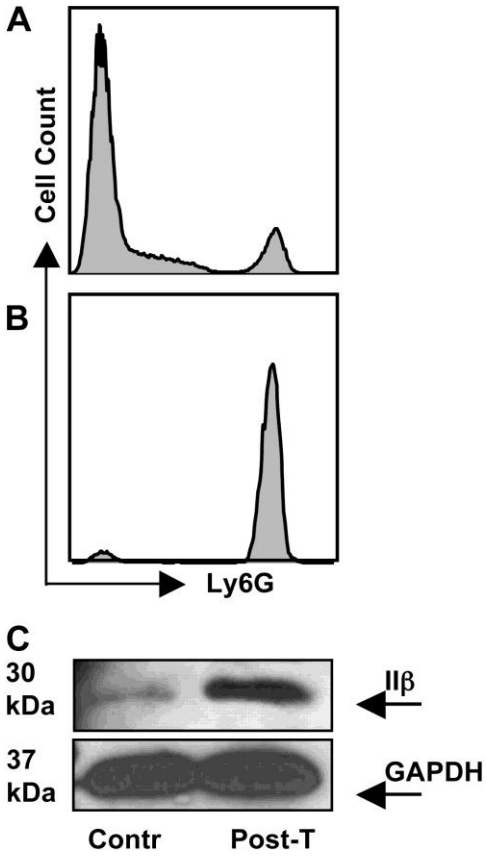


Figure 2.3. Biochemical evidence for MHC class II expression on neutrophils. (A) Neutrophils and T cells were co-cultured for 2 hr at 37°C at a ratio of 1:10, then cells were labeled with Ly6G-specific antibody. Expression of Ly6G before (A, 10% positive) and after (B, 98% positive) cell sorting for neutrophils. (C) Western blot showing MHC class IIβ expression on neutrophils after T-cell co-incubation (Post-T). C also shows GAPDH loading control blotting from the same experiment. Contr, neutrophil lysate prepared without prior T-cell incubation. These data were repeated twice with the same result.

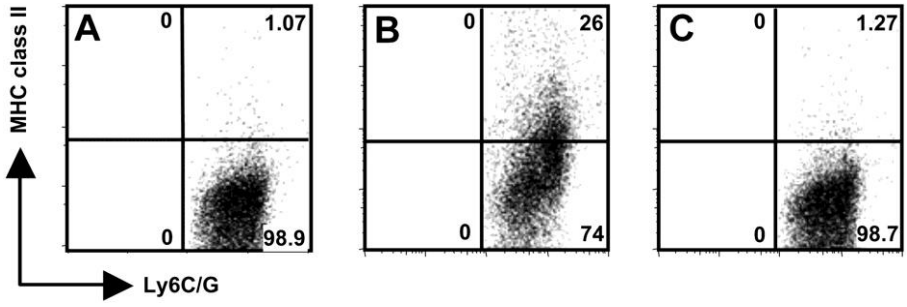


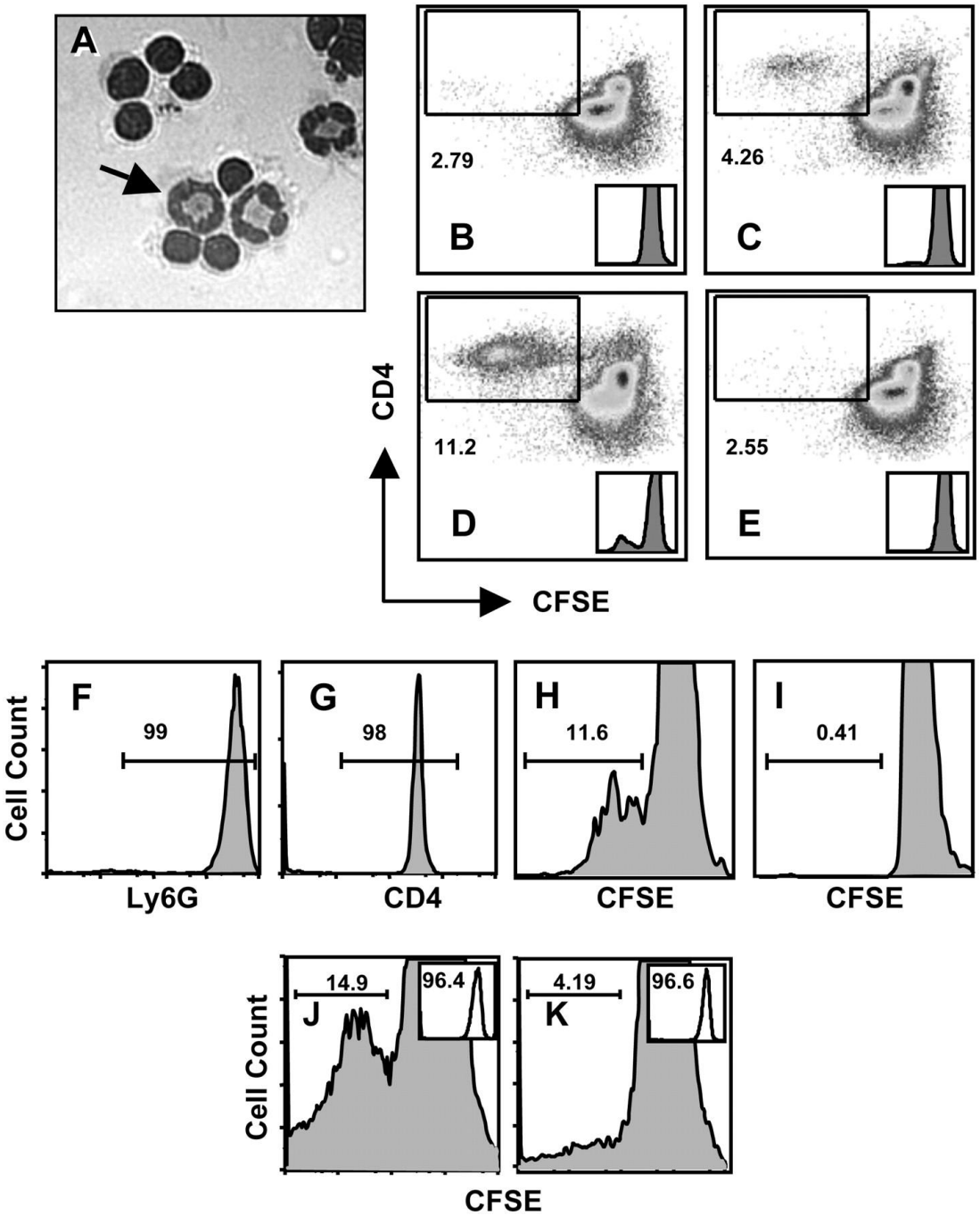
Figure 2.4. Physical contact between neutrophils and T cells is required for up-regulation of MHC class II by neutrophils. (A) Background expression of cell surface MHC class II molecules on Ly6C/G-positive neutrophils prior to incubation with T cells. (B) Expression of MHC class II molecules on the surface of Ly6C/G-positive neutrophils after 2-hr co-incubation with T cells. (C) Expression of MHC class II molecules by neutrophils when the T cells and neutrophils are separated by a Transwell membrane. The data are representative of three independent experiments.

Neutrophils process and present antigen to trigger T-cell activation

We next tested whether neutrophils expressing MHC class II molecules and co-stimulatory molecules were capable of triggering antigen-specific T cell activation. To address this question, we used $CD4^+$ T lymphocytes from OT-II mice that recognize $OVA^{323–339}$ peptide in the context of $I\text{-}A^d$. Percoll gradient-purified neutrophils were incubated with OVA antigen, washed and then added to CFSE-stained OT-II $CD4^+$ T cells and the cells were cultured for 4 days. PMN survived the 4-day co-culture with T cells, and the relative proportion at day 4 was very similar to the input proportion of T cells to neutrophils (Figure 2.5A). In contrast, neutrophils cultured in isolation rapidly lost viability (data not shown). Cells were collected, stained for CD4 and analyzed using flow cytometry to determine CFSE peak dilutions as an indicator of T cell proliferation. As expected, there was minimal proliferation when T cells were cultured with non-pulsed PMN (Figure 2.5B). In striking contrast, neutrophils pre-incubated with native OVA stimulated T cell proliferation (Figure 2.5C). Similarly, pre-incubation of PMN with $OVA^{323–339}$ peptide also triggered strong T cell proliferation (Figure 2.5D). We asked whether T cell proliferation depended upon MHC class II expression, as would be predicted for bona fide antigen presentation. As shown in Figure 2.5(E), inclusion of a blocking antibody to MHC class II completely abrogated the ability of antigen-pulsed neutrophils to trigger proliferation of OVA-specific T cells. Notably, that the activation status of isolated $CD4^+$ T cells from mice prior to incubation with neutrophils was naive, as evidenced by the expression levels of CD44, CD62L and CD25 (Figure 2.6A and C) and that these markers change expression upon activation after antigen presentation by neutrophils (Figure 2.6B and D).

Although Percoll gradient-purified PMN and immunomagnetic bead-isolated $CD4^+$ T lymphocytes were routinely >95% pure in repeated experiments, we could not completely exclude the possibility that residual macrophages or DC in the neutrophil preparations were responsible for antigen presentation, or that OVA antigen was acquired by residual APC in the T cell preparations. Therefore, to further substantiate antigen presentation by neutrophils, we repeated the experiments using high-purity cells generated by flow cytometric cell sorting. The purity of $Ly6G^+$ neutrophils and $CD4^+$ T lymphocytes is shown in Figure 2.5(F and G) and was 99 and 98% respectively. Pre-incubation of PMN with $OVA^{323–339}$ triggered robust proliferation of CFSE-labeled T cells (Figure 2.5H), while co-incubation with non-pulsed neutrophils did not induce a T cell response (Figure 2.5I).

We further addressed the possibility that residual APC in the T cell preparation were cross-presenting OVA antigen by determining the requirement for MHC class II molecules on the neutrophil population. As shown in Figure 2.5(J), OVA-pulsed PMN generated from control mice stimulated proliferation of CFSE-labeled OVA-specific $CD4^+$ T lymphocytes. However, when MHC class II-deficient PMN were used, levels of T-cell proliferation were reduced (Figure 2.5K), with the insets showing purity of the neutrophil populations used as APC. We conclude from these results that neutrophils acquire antigen and stimulate T cell proliferation in an MHC class II-dependent manner.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2.79
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CD4
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CFSE
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14.9
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Figure 2.5 (previous page). Neutrophils process and present antigen to stimulate T cell proliferation. (A) Neutrophils were pre-incubated with OVA, then cells were added to OVA-specific OT-II cells at a PMN to T cell ratio of 1: 10. Four days later, cells were examined under the microscope. The arrow points to one of three neutrophils in this image. In panels B-E, the ability of Percoll gradient-isolated neutrophils (97% purity) to stimulate immunomagnetic bead-isolated $CD4^+$ T cell (98% purity) proliferation was examined. (B) Non-pulsed neutrophils induce low levels of OT-II T cell proliferation in day 4 cultures as measured by CFSE dilution of labeled $CD4^+$ T cells. (C) Proliferation of OVA-specific OT-II T cells after day 4 co-culture with neutrophils pre-incubated (4 hr, 37°C) with whole OVA. (D) OT-II T cells proliferation after 4-day culture with OVA peptide-pulsed PMN. (E) Addition of anti-MHC class II blocking antibody (M5/114) prevents OT-II proliferation stimulated by OVA peptide-pulsed neutrophils. Insets in B through E show CFSE peak dilution histograms. Samples in B through E were incubated at a ratio of 10 T-cells for every one neutrophil. In panels F-I, the experiments were reiterated using flow sorted $Ly6G^{high}$ neutrophils and $CD4^+$ OT-II T cells. (F) Neutrophil purity after cell sorting (99% Ly6G positive). (G) Purity of OT-II T cells following cell sorting (98% CD4 positive). (H) Day 4 proliferation of sorted CFSE-labeled OT-II $CD4^+$ T cells after incubation with sorted neutrophils pulsed with OVA peptide. (I) Proliferation of sorted OT-II T cells after incubation with non-pulsed sorted neutrophils. (J) Proliferation of OVA-specific OT-II T cells following incubation with OVA peptide-pulsed neutrophils from MHC class II expressing C57BL/6 mice. (K) Proliferation of $CD4^+$ T cell following 4-day incubation with OVA peptide-pulsed neutrophils from MHC class II-deficient mice. Neutrophil purity, based on Ly6G expression, is shown in the inserts and was 96.4 and 96.6% in J and K, respectively. Samples in (F) through (K) were incubated at a ratio of five T-cells for every one neutrophil. Experiments in

(A-D) were performed five times with the same result and the MHC blocking experiment (E) was performed three times with the same result. The cell sorting experiment (F-I) was performed three times and experiments with MHC class II KO cells were performed two times with the same result.

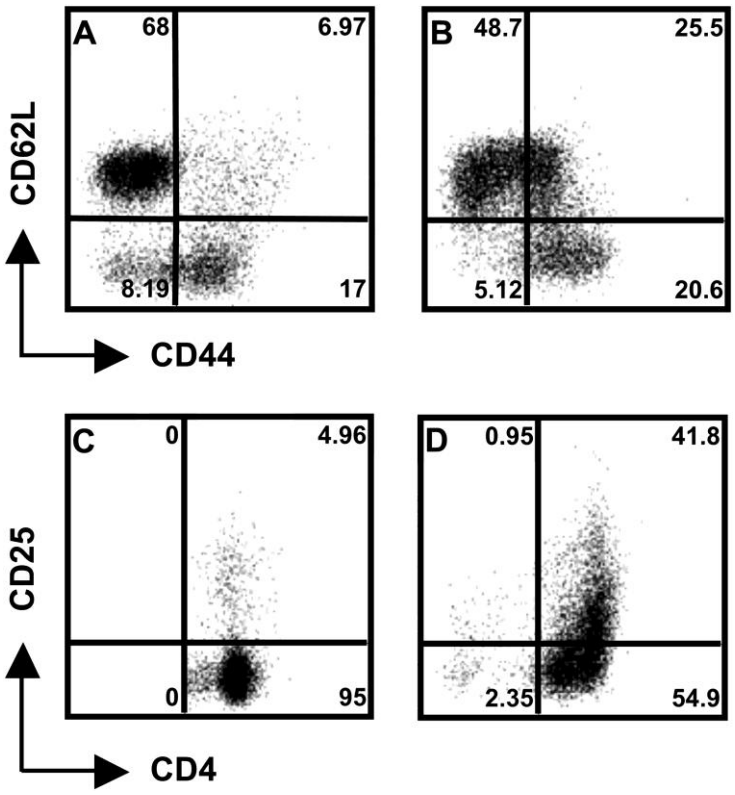


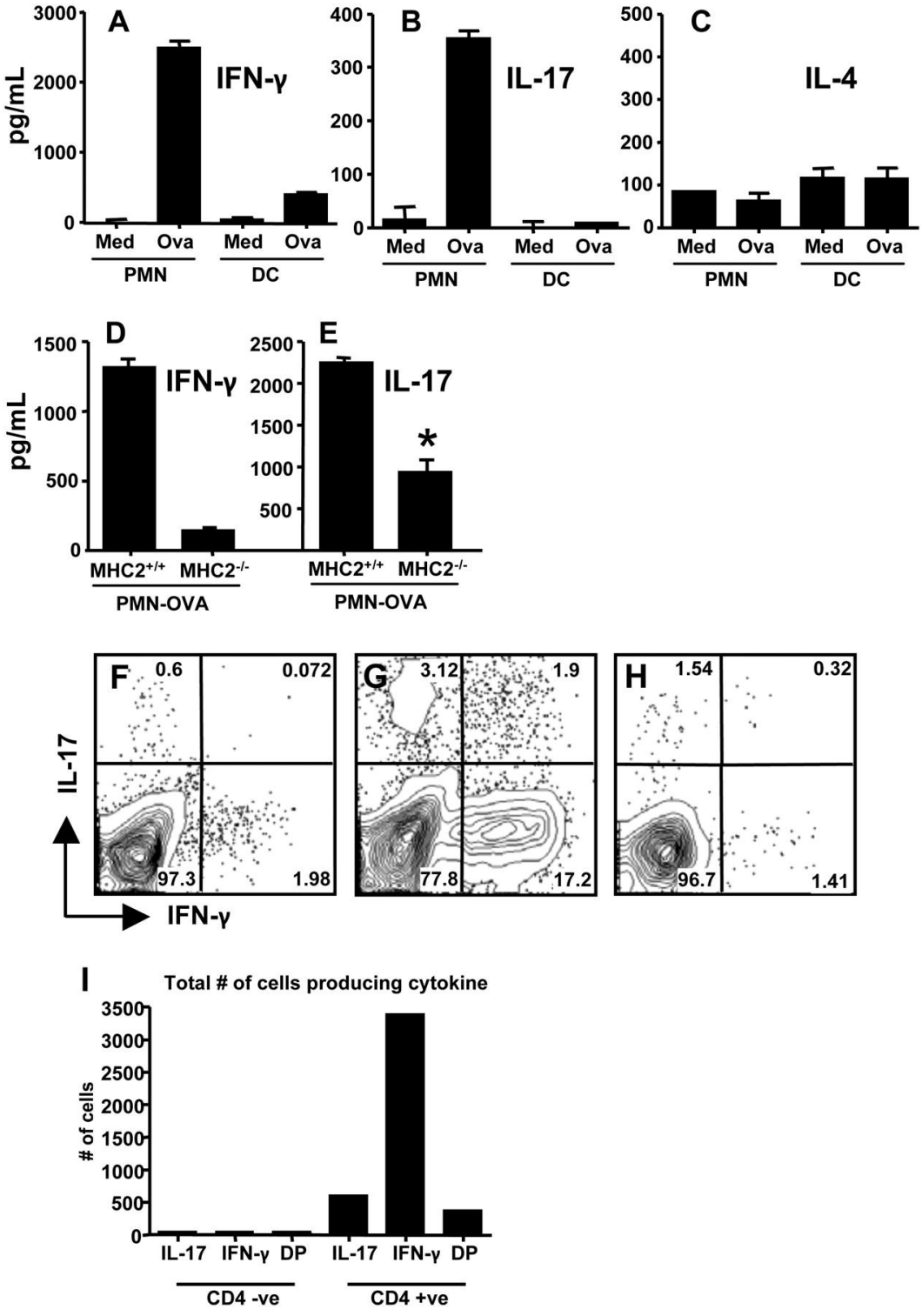
Figure 2.6. OTII CD4$^+$ T cells exhibit a naive phenotype prior to antigen presentation by neutrophils. Expression of CD44 and CD62L by CD4$^+$ T cells from a naive spleen of an OT-II mouse (A) and expression of these markers after neutrophils antigen presentation in (B). Expression of CD25 on CD4$^+$ T cells before and after neutrophils antigen presentation is shown in (C and D), respectively. The numbers indicate the relative percentage of cells falling within each indicated gate.

Neutrophils induce differentiation of T_h1 and T_h17 cells

Given that OVA-pulsed PMN triggered antigen-specific T cell proliferation, we were interested in the type of immune response generated. Therefore, we measured cytokine levels in supernatants from PMN and OVA-specific T cell co-cultures and compared these with responses in co-cultures of T cells and DC. Surprisingly, even without addition of exogenous cytokines, OVA-pulsed, but not control, neutrophils stimulated production of IFN-γ (Figure 2.7A) and IL-17 (Figure 2.7B). In contrast, parallel cultures of OVA-pulsed DC and T cells displayed minimal IFN-γ and no detectable IL-17. Neither PMN nor DC triggered production of IL-4 in T cell co-cultures (Figure 2.7C). We then examined if cytokine production stimulated by OVA-pulsed neutrophils, like the T cell proliferative response, required PMN expression of MHC class II molecules. As predicted, IFN-γ (Figure 2.7D) responses were highly dependent upon neutrophil MHC class II molecule expression. For the case of IL-17 (Figure 2.7E), there was a major reduction in cytokine production using MHC class II-negative neutrophils, but some IL-17 production clearly remained. The basis for this residual response is not clear. One possibility is that neutrophils, which are known to sometimes produce IL-17 (24, 25), release the cytokine in response to T cell co-culture. It is also possible that OVA-pulsed PMN produce factors that trigger MHC class II-independent T cell IL-17 production.

We also performed intracellular cytokine staining on $CD4^+$ T cells following incubation with OVA-pulsed neutrophils. As shown in Figure 2.7(F), there was minimal IFN-γ and IL-17 expression in T cells after co-culture with non-pulsed neutrophils. However, as shown in Figure 2.7(G), incubation with OVA-pulsed PMN stimulated generation of both T_h1 and T_h17 T cells as defined by production of IFN-γ and IL-17, respectively. Interestingly, we also detected a small

population of T cells expressing both of these cytokines. Notably, production of these cytokines is largely restricted to $CD4^+$ T cells as gating on the $CD4^-$ population shows a minimal percentage of cytokine being produced (Figure 2.7H), and when plotted as total number of cytokine producing cells, it is evident that the cytokines are produced by $CD4^+$ T cells (Figure 2.7I). The cytokines IL-6 and TGF-β together are known to drive T_h17 differentiation. Therefore, we used blocking mAb to ask whether these cytokines were involved in neutrophil-induced generation of T_h17 cells. Surprisingly, presence of these antibodies had no effect on the PMN-induced IL-17 response (Figure 2.8A). In contrast, and as expected, the same antibodies were capable of blocking skewing toward IL-17 responses induced by bone marrow-derived DC cultured with IL-6 and TGF-β (Figure 2.8B). Thus, antigen-pulsed neutrophils are capable of stimulating differentiation of both T_h1 and T_h17 T cells even without the addition of exogenous polarizing cytokines.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IL-4
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Figure 2.7 (previous page). OVA-pulsed neutrophils drive differentiation of T_h1 and T_h17 cells *in vitro*. (A-C) PMN and bone marrow-derived DC were pulsed with OVA peptide or medium alone (Med) then cultured with OVA-specific T cells. At day 4 after culture initiation, supernatants were collected and assayed for IFN-γ (A), IL-17 (B) and IL-4 (C). In (D and E) similar cultures were initiated using neutrophils from wild-type and MHC class II knockout mice. Day 4 supernatants were tested for IFN-γ (D) and IL-17 (E, where * indicates $P<0.001$). In panels (F and G), the co-cultures were subjected to intracellular cytokine staining following incubation with non-pulsed (F) and OVA peptide-pulsed (G) neutrophils. The results in F and G show intracellular IFN-γ and IL-17 after gating on $CD4^+$ T cells. Panel (H) shows intracellular staining on CD4-negative gated population, while panel (I) shows the total number of cells staining positive for IL-17, IFN-γ or both (DP, double positive) in both the CD4 negative and positive populations. These experiments were performed twice with the same result.

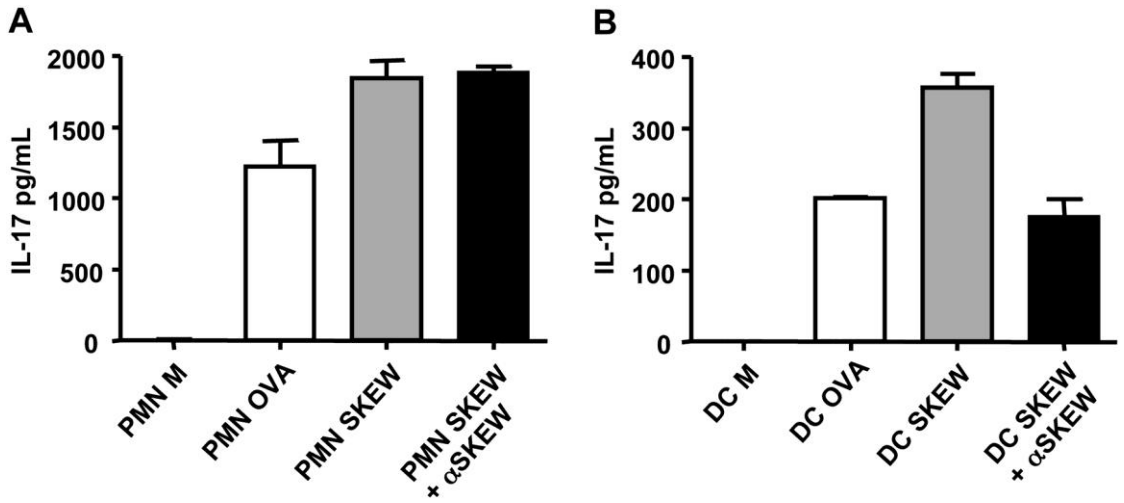

Figure 2.8. T_h17 induction by neutrophils is both IL-6 and TGF-β independent. Purified neutrophils (A) and bone marrow-derived DC (B) were pulsed with OVA peptide and incubated with OVA-specific $CD4^+$ T cells for 4 days in the presence of a T_h17 skewing cocktail. In the indicated samples, an anti-skew mAb cocktail was included. On day 4, cultures were spun down and fresh medium supplemented with IL-2 added to co-cultures. Supernatant was collected 3 days after fresh media addition. Cytokine IL-17 levels were measured by ELISA. M, medium only; Skew, IL-17 skewing cocktail consisting of IL-6 (20 ng ml^{-1}), TGF-β (1 ng ml^{-1}), anti-IFN-γ (10 μg ml^{-1}) and anti-IL-12 (10 μg ml^{-1}); αSkew, anti-skewing cocktail consisting of anti-IL6 and anti- TGF-β mAb each at 10 μg ml^{-1}.

Discussion

Neutrophils are conventionally viewed as short-lived cells that migrate rapidly and in large number to sites of infection and inflammation (1). Here, they phagocytose particulate antigen and release granule-associated microbicidal mediators, then rapidly undergo apoptotic death. The response is completed when tissue macrophages and DC phagocytose apoptotic PMN, thereby contributing to resolution of infection and inflammation (26, 27). Yet, it is known that neutrophils respond to inflammatory cytokines by producing immunoregulatory mediators and delaying their own apoptotic death, suggesting a more active role for PMN during infection (28-30). The results of the present study unequivocally demonstrate that neutrophils express MHC class II molecules that directly present antigenic peptide, induce T cell proliferation and promote generation of T_h17 effector cells. MHC class II molecules were not constitutively expressed by neutrophils, but instead up-regulation of these proteins required contact with T cells. Therefore, the present results reinforce an emerging view of PMN as active orchestrators of innate and adaptive immunity (1).

Our data show that OVA-pulsed neutrophils are programmed to induce T_h17 differentiation even without addition of exogenous cytokines. This would appear to be an important, and possibly unique, property of PMN. Other APC, such as DC, typically require addition of recombinant cytokines to mediate optimal T-lymphocyte subset differentiation during cell culture. T_h17 cells are now understood to be an independent T-cell lineage whose differentiation is controlled by TGF-β and IL-6 (31-33). T_h17 cells are implicated in autoimmunity and inflammation associated with several diseases, including Crohn's disease in humans and experimental autoimmune encephalitis in mice (31). The cells are also important in host defense, insofar as they have been

shown to play a protective role during infection with extracellular pathogens such as *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Candida albicans* (34-36).

A key property of IL-17, the signature cytokine of T_h17 cells, is its ability to promote neutrophil recruitment and granulopoiesis. This is mediated by chemokines such as macrophage inflammatory protein-2 and growth factors such as granulocyte colony-stimulating factor and stem cell factor (36). IL-17 is also known to potentiate neutrophil cytotoxic and phagocytic activity (37, 38). Our results reveal an amplification loop in which antigen-loaded PMN induce T_h17 generation; production of IL-17, in turn, can be expected to promote increased neutrophil activity. Further evidence for cross talk between neutrophils and T_h17 cells is provided by data showing mutual chemoattraction between these cell types mediated by reciprocal expression of chemokines and chemokine receptors (39).

While neutrophils can produce IL-12 that drives T_h1 generation (3, 4), the neutrophil factors responsible for T_h17 induction are not yet known. In this regard, it has been reported that apoptotic PMN favor T_h17 generation. This is mediated through IL-6 and TGF-β elaboration by APC phagocytosing apoptotic neutrophils (40). Whether a similar pathway is involved in neutrophil-dependent T_h17 generation described here is not known. However, we found that blocking antibodies specific for IL-6 and TGF-β failed to affect T_h17 generation driven by OVA-pulsed PMN, arguing that these cytokines are not involved in neutrophil driven T_h17 induction. We are currently pursuing the possibility that presently undefined neutrophil mediators induce differentiation of this T cell subset independently of TGF-β or IL-6.

Neutrophils are not generally known for their ability to serve as professional APC. Nevertheless, some studies provide evidence that PMN can be induced to express MHC class II and co-stimulatory molecules. For example, both precursor and mature human PMN up-regulate MHC class II molecules following stimulation with either GM-CSF, IFN-γ or IL-3 (10, 12, 41). It has also been reported that cross-linking of neutrophil CD11b results in up-regulation of MHC class II molecules on PMN (42, 43). Unlike several cytokines secreted by neutrophils (44-46), expression of MHC class II molecules results, at least in part, from up-regulation of mRNA synthesis, rather than exocytosis of preformed MHC class II protein from intracellular granules (11). Neutrophil MHC class II expression has also been reported at the site of inflammation in rheumatoid arthritis, in Wegener's granulomatosis and in persistent *Staphylococcus aureus* infection (17, 47, 48). Similar to MHC class II molecules, the co-stimulatory molecules CD80 and CD86 have been shown to be up-regulated on neutrophils in response to inflammatory cytokines and during autoimmune pathology (16, 17, 42).

Despite this evidence, the functional consequences of inducible MHC class II and co-stimulatory CD80/CD86 on neutrophils has been unclear. Using human PMN, it has been shown that these cells stimulate T cell proliferation in response to the superantigens *Staphyloccocal enterotoxin* A and E (11, 19). In related experiments, neutrophils have been found to process exogenous bacterial antigens for MHC class I-restricted presentation to $CD8^+$ T lymphocytes (49). These cells could also secrete processed peptide that was subsequently acquired by neighboring macrophages or DC. More directly related to our studies, it was recently shown that mouse neutrophils loaded with antigenic OVA peptide stimulated proliferation and cytokine secretion by antigen-specific $CD4^+$ T cells (20). Our results are significant insofar as they are the first to

demonstrate a neutrophil capability to actively process and present peptide antigen to T cells and to simultaneously trigger T_h17 differentiation.

In addition to their effects on T cells, there is clear evidence that PMN exert immunoregulatory effects on DC (8). Neutrophils release chemokines that attract both DC and T cells and they release cytokines that trigger DC co-stimulatory molecule expression as well as IL-12 and TNF-α secretion (50-53). This is mediated through physical contact mediated by $Lewis^X$ carbohydrate moeities on PMN Mac-1 and DC-SIGN expressed by DC, combined with neutrophil release of TNF-α (54). Neutrophils also release several mediators during degranulation or apoptosis, and many of these serve as 'alarmins' that mobilize and activate APC (55). Among the alarmins produced by neutrophils are the human α-defensins HNP-1 and HNP-2 that chemoattract immature DC and naive T cells (53). Notably, these peptides function as adjuvants when administered to mice with OVA (56). Thus, neutrophils possess the ability to activate DC as well as directly trigger antigen-specific T cell immunity during infection.

The concept that a single APC can present antigen to T cells and provide polarizing signals that drive T-cell subset differentiation has received considerable attention. DC are well known to serve this role during T_h1 induction. Recently, it was proposed that IL-4-producing basophils can function similarly in presenting antigen and stimulating T_h2 differentiation (57-59). Our results suggest that neutrophils are a third type of APC that possesses a parallel ability to directly stimulate naive T cells and instruct differentiation to the T_h1 and T_h17 effector T cell subsets, a finding with broad implications with regard to disease pathogenesis and control of infection.

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CHAPTER 3

Toxoplasma gondii Triggers Release of Human and Mouse Neutrophil Extracellular Traps*

* Reprinted from Delbert S. Abi Abdallah, Changyou Lin, Carrissa J. Ball, Michael R. King, Gerald E. Duhamel and Eric Y. Denkers. *Toxoplasma* gondii Triggers Release of Human and Mouse Neutrophil Extracellular Traps. Submitted 2011

Abstract

Neutrophils have recently been shown to release DNA-based extracellular traps that contribute to microbicidal killing and have also been implicated in autoimmunity. The role of neutrophil extracellular trap (NET) formation in the host response to nonbacterial pathogens has received little attention and remains largely unexplored. Here, we show for the first time that the protozoan pathogen *Toxoplasma gondii* elicits production of NETs from human and mouse neutrophils. Tachyzoites of each of the three major parasite strain types were efficiently entrapped within NETs, resulting in decreased parasite viability. We also show that *Toxoplasma* activates a MEK-ERK pathway in neutrophils and that inhibition of this pathway leads to decreased NET formation. To determine if *Toxoplasma* induced NET formation *in vivo* we employed a mouse intranasal infection model. We found that administration of tachyzoites by this route induced rapid tissue recruitment of neutrophils with evidence of extracellular DNA release. Taken together, these data indicate a role for NETs in the host innate response to protozoan infection. We propose that NET formation limits infection by direct microbicidal effects on *Toxoplasma* as well as by interfering with the ability of the parasite to invade target host cells.

Introduction

Neutrophils have long been regarded as one of the most important of the induced host innate defenders primarily because they are the earliest cells to arrive at sites of infection or inflammation in response to chemotactic signals. They can rapidly accumulate in large numbers and deploy a diverse arsenal of weapons aimed at eliminating invading pathogens. Resolution of

inflammation and infection involves apoptosis of neutrophils and phagocytic elimination by macrophages. As such, neutrophils are often regarded as potent but short-lived cells with limited ability to affect adaptive immunity (1). Nonetheless, their biological significance may extend beyond this view insofar as neutrophils can also display immunoregulatory activity on other cells of the immune system (1-6).

Neutrophils engulf pathogens through phagocytosis, and the resulting microbe-carrying phagosome fuses with lysosomes where the microorganism can be degraded. Microbial killing relies on both oxidative and non-oxidative mechanisms. Oxidative mechanisms involve production of reactive oxygen species through the activity of the NADPH oxidase enzyme complex, while non-oxidative mechanisms rely on the release of antimicrobial peptides and proteases (7, 8). Together, these mechanisms were until recently thought to encompass the whole antimicrobial activity of neutrophils.

A landmark study by Brinkmann et al. identified a previously unrecognized neutrophil anti-microbial mechanism involving release of nuclear DNA that can entrap and kill extracellular pathogens (9). Originally discovered in neutrophils, formation of extracellular traps has now been described in eosinophils and mast cells (10, 11). Neutrophil extracellular traps (NETs) have been shown to be composed of a DNA backbone studded with histones and laced with various anti-microbial peptides that together can kill microbial pathogens. Several bacterial and fungal pathogens have been shown to be susceptible to NET killing, including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Escherichia coli*, *Mycobacterium*

tuberculosis, *Listeria monocytogenes* and *Candida albicans* (9, 12-16). There is also evidence that some bacterial pathogens avoid killing by NETs by releasing nucleases (12, 13). At present, much less is known about the extent to which neutrophils form NETs in response to protozoan pathogens, although there is recent evidence for *Leishmania*-induced NET formation (17, 18).

The ability of another major protozoan parasite, *Toxoplasma gondii*, to elicit neutrophil recruitment during infection raises the question of whether or not NET formation is elicited by this important human opportunistic pathogen. *Toxoplasma* normally causes asymptomatic infection in immunocompetent individuals but the parasite can cause serious clinical disease in immunocompromised hosts (19, 20). Immunity to *T. gondii* consists of a strong T_h1 response that provides protection to the host. However, this inflammatory response can become pathological if not appropriately controlled by down-modulatory cytokines (21, 22). There is evidence that neutrophils play a role during *Toxoplasma* infection, inasmuch as they are rapidly recruited to the site of infection, lack of recruitment in the absence of CXCR2 correlates with increased susceptibility, and they are capable of producing several cytokines and chemokines in response to the parasite (23-26). The ability of *Toxoplasma* to elicit formation of NETs has not been addressed.

Here, we show for the first time that both murine and human neutrophils release NETs in response to all three major clonal lineages of *Toxoplasma*, and that the parasites become physically entrapped by these macromolecular structures. Formation of NETs (also called NETosis) is invasion independent, but partially depends upon activation of ERK1/2 MAPK. In a

mouse intranasal infection model that stimulates rapid neutrophil accumulation into the lungs, we provide in vivo evidence for release of NETs in response to the parasite. We hypothesize that NET release by neutrophils functions as an innate mechanism of parasite killing, and that by trapping parasites, NETs interfere with the ability to infect host cells and establish infection.

Materials and Methods

Mice

Female C57BL/6 and Swiss Webster mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) or Taconic Farms (Germantown, NY, USA) and used at 6-8 wk of age. C57BL/6 LYS-eGFP knock-in mice were a generous gift from David Sacks (National Institutes of Health). All mice were maintained in the Transgenic Mouse Core Facility at the Cornell University College of Veterinary Medicine, which is accredited by the American Association for Accreditation of Laboratory Animal Care. All animal experiments were approved by the Cornell University Institutional Animal Care and Use Committee.

Mouse neutrophil isolation

Mouse neutrophils were isolated as previously described (27). Briefly, mice were i. p. injected with 0.5 ml of 10% thioglycollate (Becton Dickinson, Franklin Lakes, NJ, USA) and 20 hr later, peritoneal exudate cells (PEC) were collected by peritoneal cavity lavage with PBS (Cellgro, Manassas, VA, USA). Neutrophils were subsequently purified by continuous Percoll gradient centrifugation as described elsewhere (28). Briefly, Percoll (GE Healthcare, Fairfield, CA,

USA), adjusted to pH 7.4, was mixed at a ratio of 1:9 with PEC resuspended in PBS. The mixture was then transferred to a 10 ml polycarbonate centrifuge tube and ultracentrifugation was performed at 60,000 x g for 65 min at 4°C using a 50 Ti rotor (Beckman Centrifuges, Brea, CA, USA). The layer enriched for neutrophils was washed twice with PBS and resuspended in complete DMEM (cDMEM), consisting of 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 30 mM HEPES (all purchased from Invitrogen Life Technologies, Carlsbad, CA, USA), 2% bovine growth serum (Hyclone, Logan, UT, USA), and 0.05 mM β-mercaptoethanol in DMEM. Neutrophil preparations were routinely over 95% pure.

Human neutrophils

Human neutrophils were purified as described previously (29). Briefly, human peripheral blood was collected from healthy adult donors following informed consent. Neutrophils were isolated by centrifugation at 500 x g for 50 min at 23°C in a Sorvall ST 16 Centrifuge (Fisher Scientific, Pittsburgh, Pennsylvania) using 1-Step Polymorphs (Accurate Chemical and Scientific Corporation, Westbury, NY, USA.) The neutrophil layer was collected and washed twice in Ca^{2+} and Mg^{2+} free HBSS and any remaining red blood cells were subjected to hypotonic lysis by brief incubation in DPBS: endotoxin-free water (ratio of 1:6) followed by osmotic restabilization with 4 x DPBS. The cells were resuspended in HBSS containing 0.5% HSA, 2mM Ca^{2+}, 10 mM HEPES, buffered to pH 7.4.

HL60 differentiation

HL60 differentiation was performed as described earlier (30). Briefly, HL-60 cells (4 x 10^5 cells ml^{-1}) were treated with 1.25% dimethylsulfoxide (DMSO, Calbiochem) in RPMI 1640 supplemented with 5% heat-inactivated FBS for 4 days. Differentiated cells were harvested by centrifugation at 500 x g for 10 min at room temperature, and washed once with medium used in NET experiments.

Picogreen DNA measurement

Extracellular DNA was quantitated using a previously described method (31). Briefly, 2 x 10^5 neutrophils were seeded in triplicate into a 96-well cell culture plate (Costar, Corning, NY, USA). Medium, PMA or parasites were added to the cells and the plate was briefly centrifuged to synchronize infection. At the indicated time points, 50 μl of 500 mU ml^{-1} micrococcal nuclease (Worthington Biochemical, Lakewood, NJ, USA) was added and the culture was incubated for 10 min at 37°C. Enzymatic digestion was terminated using 5 mM EDTA and cultures were centrifuged at 200 x g for 8 minutes. 100 μl of the cell free supernatant was transferred to a flat bottom 96-well plate for quantification of double-stranded DNA using the Quant-iT Picogreen assay (Invitrogen, Carlsbad, CA, USA). 100 μl of Picogreen reagent was added to the samples which were then incubated at room temperature in the dark for 4 min. Extracellular DNA was measured in a spectrofluorometer at 480 nm excitation and 520 nm emission.

Sytox Green viability assay

Mouse neutrophils with parasites tagged with Tomato Red protein (generated by Dr. B. Striepen, University of Georgia and kindly provided by Dr. E. Robey, University of California-Berkeley) in the presence of 1 μM cytochalasin D (Calbiochem, Darmstadt, Germany) to inhibit parasite invasion. After 4 hr at 37°C, media was aspirated and coverslips incubated with Sytox Green live cell exclusion dye (Invitrogen, Carlsbad, CA, USA) for 10 min at room temperature. Coverslips were then rinsed in PBS, mounted using ProLong Antifade containing 4′,5-diamidino-2-phenylindoleI (Molecular Probes, Carlsbad, CA, USA) and visualized by fluorescence microscopy.

ERK1/2 inhibition

To inhibit ERK phosphorylation, cells were pretreated with 10 μM of the MEK 1/2 inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene; Cell Signaling Technology, Danvers, MA, USA) for 2 hr at 37°C. Parasites (MOI 5:1) or PMA (60 nM) were then added to the cells and samples were subsequently collected for fluorescence microscopy or were lysed with SDS sample reducing buffer for Western blot analysis.

Western blotting

Cell lysates were resolved by SDS-PAGE, and immunoblotted using an anti-phospho-p44/42 MAPK (ERK1/2) and anti-phospho-p38 Ab (Cell Signaling Technology, Danvers, MA, USA). Blots were stripped and re-probed for total p44/42 ERK and p38 antibodies (Cell Signaling).

Blots were visualized using an ECL-based detection system (Thermo Scientific, Rockford, IL, USA).

Immunofluorescence microscopy

Neutrophils were incubated on poly-L-lysine treated glass coverslips in a 24-well plate with medium, PMA or *Toxoplasma*, centrifuged for 5 min at 200 x g to initiate infections, followed by incubation at 37°C for the indicated times. Samples were collected by gently removing coverslips, fixing with 3% paraformaldehyde (20 min, room temperature), then blocked in PBS supplemented with normal mouse serum and fetal calf serum for 1 hr at room temperature. Coverslips were incubated with FITC-conjugated anti-*Toxoplasma* SAG-1 Ab (Argene, Massapequa, NY, USA), then washed and mounted with ProLong Antifade containing 4′,5-diamidino-2-phenylindoleI (Molecular Probes). Staining with anti-histone H3 Ab was accomplished according to the manufacturer's instructions (Cell Signaling Technology). Briefly, coverslips were fixed as described above, then permeabilizated using ice-cold 100% methanol for 10 min at -20°C followed by washing in PBS. Coverslips were blocked for one hr at room temperature then incubated overnight with anti-histone H3 Ab. Coverslips were then washed three times with PBS and incubated with Alexa Fluor-647 goat-anti-rabbit IgG (H+L) Ab (Invitrogen, Carlsbad, CA, USA) for 2 hr at room temperature. After rinsing in PBS, coverslips were mounted with ProLong Antifade containing DAPI. Images were collected with an Olympus BX51 fluorescence microscope equipped with a DP 70 camera using Olympus DP controller software and Olympus DP manager software or an SP5 Leica confocal microscope.

Mouse intranasal infection model

5 x 10^7 RH strain parasites in PBS were administered in a total volume of 50 µl intranasally to Swiss Webster mice. Control mice were intranasally administered the same volume of sterile PBS. Mice were euthanized 6 hr post-infection and lungs were perfused with 3% paraformaldehyde through tracheal injection. Lungs were then excised and further fixed by immersion in 3% paraformaldehyde overnight. Lungs were then paraffin embedded and sectioned for staining. Bronchoalveolar lavage fluid (BALF) was collected by washing lungs with 200 µl of PBS twice. Samples were spun down at 1000 r.p.m for 8 minutes and supernatants assessed for dsDNA content using the Picogreen assay described above.

Statistical analyses

Statistical analyses were performed using the Prism Software. Two way ANOVA unpaired tests with Turkey's post test analysis and One way ANOVA tests with Bonferroni post test analysis were used to determine statistical significance. *P* values<0.05 were considered significant.

Results

Mouse neutrophils undergo NETosis in response to PMA

Most studies to date that examine NET formation have employed human neutrophils (9, 17, 32, 33). We wanted to determine whether mouse PMN could also release NETs after appropriate stimulation, and in particular in response to *T. gondii*. We used as a cell source thioglycollate-elicited, Percoll gradient-purified mouse neutrophils (27). In addition, we employed cells isolated

from LYS-eGFP knock-in mice in which GFP-tagged lysozyme is constitutively expressed. The reasoning for this was that it has been shown that lysozyme is retained inside cells undergoing NETosis (34). Since NETs are composed of a DNA backbone with histone molecules studded across the DNA fibers, we stained for both DNA and histones as a positive identifier of NET formation. As shown in Figure 3.1A, neutrophils incubated in medium alone did not spontaneously extrude extracellular traps, as determined by absence of staining for extracellular DNA and histone H3. However, we show in Figure 3.1B that neutrophils incubated with PMA for 4 hrs formed extracellular traps, as determined by release of DAPI-positive strand-like material (panel a) that also stained positive for histone H3 (panel b). Further confirmation for NETosis was that, as predicted, there was no concurrent release of intracellular lysozyme (c). To quantify NET formation over time, cells were stimulated with PMA then they were treated at specific time points with micrococcal nuclease to solubilize extracellular DNA. Cell-free supernatants were tested for nucleic acid content using PicoGreen, a fluorochrome that binds specifically to double-stranded DNA. As is shown in Figure 3.2A, PMA induced robust release of DNA, which could be detected within 2 hr of PMN stimulation.

Toxoplasma elicits NET formation by mouse neutrophils

To determine whether *Toxoplasma* triggers release of extracellular traps from mouse neutrophils, we incubated thioglycollate-elicited PMNs with tachyzoites and determined NET formation 4 hr later. As shown in Figure 3.3A-D, co-incubation with the Type I lineage RH strain stimulated formation of NETs, as determined by DNA release (panel A) and staining for histone H3 (panel B). Furthermore, staining for *Toxoplasma* (panel C and D) showed evidence for parasite

entrapment within the NETs. As with neutrophil stimulation with PMA (Figure 3.2A), rapid release of double-stranded DNA during co-incubation with tachyzoites could also be quantitatively measured by PicoGreen binding (Figure 3.2B). There are 3 major clonal lineages of *T. gondii* that differ in host responses elicited (35, 36), prompting us to ask whether they differ in ability to induce NETs. However, we found that like the Type I RH strain (Figure 3.3A-D), both Type II (Figure 3.3E and F) and Type III (Figure 3.3G and H) parasite strains triggered release of NETs in which parasites appeared to be entrapped.

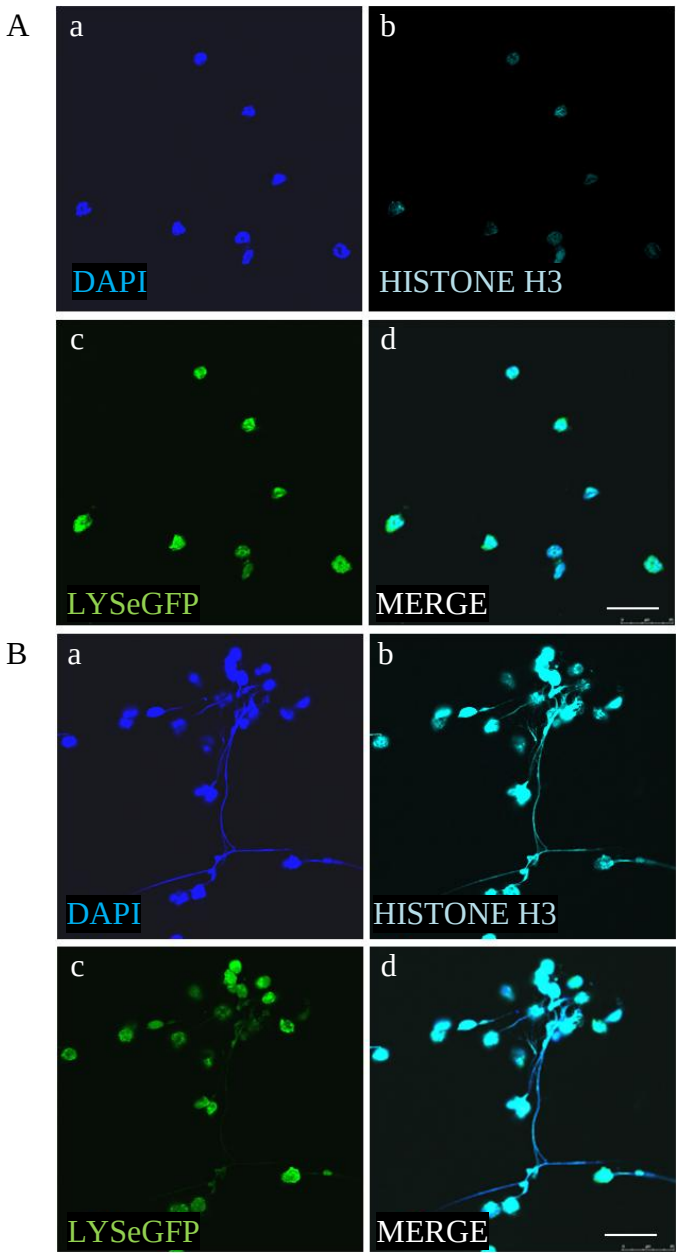


Figure 3.1. Thioglycollate-elicited mouse neutrophils produce NETs in response to PMA. Purified PMN from Lys-eGFP mice were incubated for 4 hr in medium alone (A) or medium with 600 µM PMA (B). NETs were visualized by staining with DAPI (a) and histone H3 (b). Lysozyme was retained within cells confirming that NET formation was a regulated event rather than non-specific cell lysis (c). Merged images are shown in d. The scale bar indicates 20 µm. These experiments were repeated at least 3 independent times with similar results.

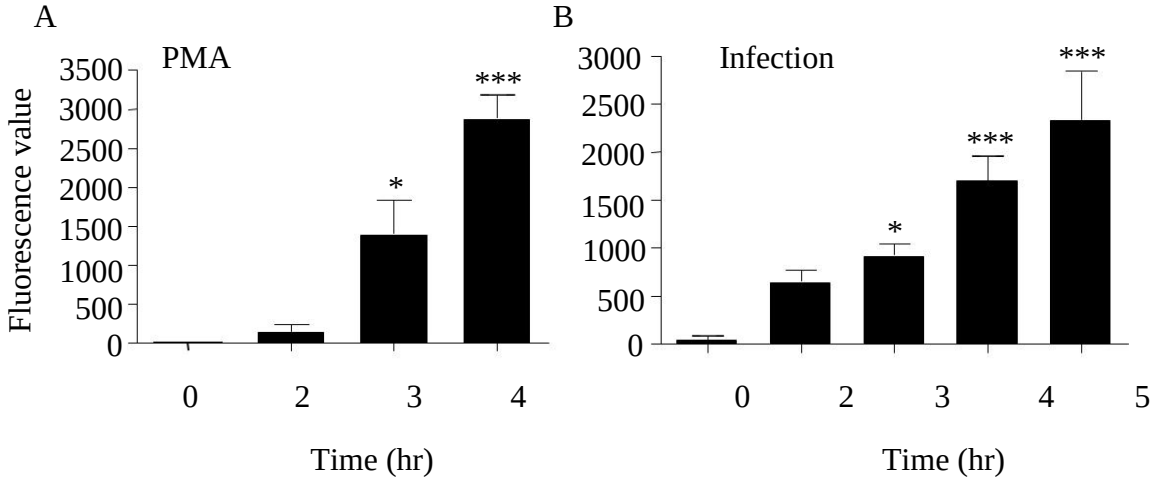
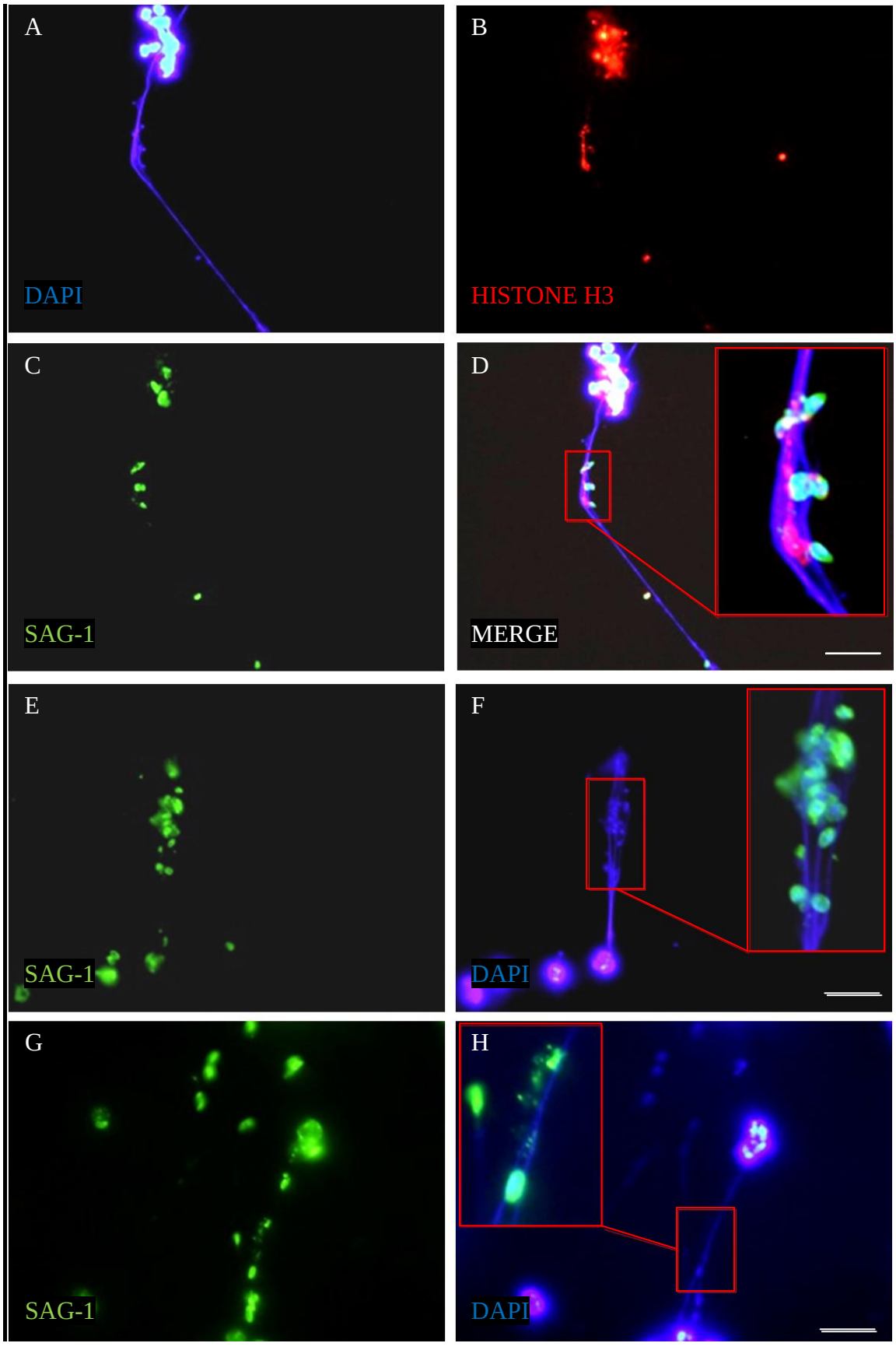


Figure 3.2. Quantitative measurement of PMA and *Toxoplasma*-induced NET formation. Mouse neutrophils were either stimulated with 600 μM PMA (A) or infected with RH strain tachyzoites (MOI 5:1) and NET formation was measured by solubilizing extracellular DNA and measuring PicoGreen fluorescence in supernatants as an indicator of double-stranded DNA. Data for both experiments have been corrected to account for contribution to the fluorescence signal by either cells incubated with medium alone or parasites incubated with medium alone. Experiments were repeated at least 3 independent times with similar results. * $p < 0.05$, ** $p < 0.01$ relative to time-point zero.


A
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B
HISTONE H3
C
SAG-1
D
MERGE
E
SAG-1
F
DAPI
G
SAG-1
H
DAPI

Figure 3.3. Release of NETs occurs in a parasite strain-independent manner. Purified mouse neutrophils were added to Type I (RH; A-D), II (PTG, E and F) and III (CTG; G and H) strains of *Toxoplasma* and NET formation was assessed 4 hr later by staining for DNA (A, F and H) and histone H3 (B). Tachyzoites were visualized by staining with Ab to parasite surface protein SAG-1 (C, E, and G). The insets in panels D, F, and H are merged and expanded images showing entrapment of parasites within NETs. The scale bars indicate 20 μm. Experiments were repeated at least 3 independent times with similar results.

Active invasion is not required for NET formation

We envisioned that *Toxoplasma* could stimulate NET release during invasion of host cells, or that neutrophil DNA release could be triggered by soluble factors derived from extracellular parasites. To distinguish between these possibilities, we asked whether NET formation occurred in the presence of cytochalasin D, a drug blocking actin polymerization that is required for parasite entry into the host cell (37). Figure 3.4A shows formation of NETs when co-incubation with parasites (shown in Figure 3.4B) is carried out in the presence of cytochalasin D. The merged image shown in Figure 3.4C also reveals clear evidence of parasite entrapment within NETs (Figure 3.4C). We conclude that invasion of host neutrophils is not required for NET induction by *T. gondii.*

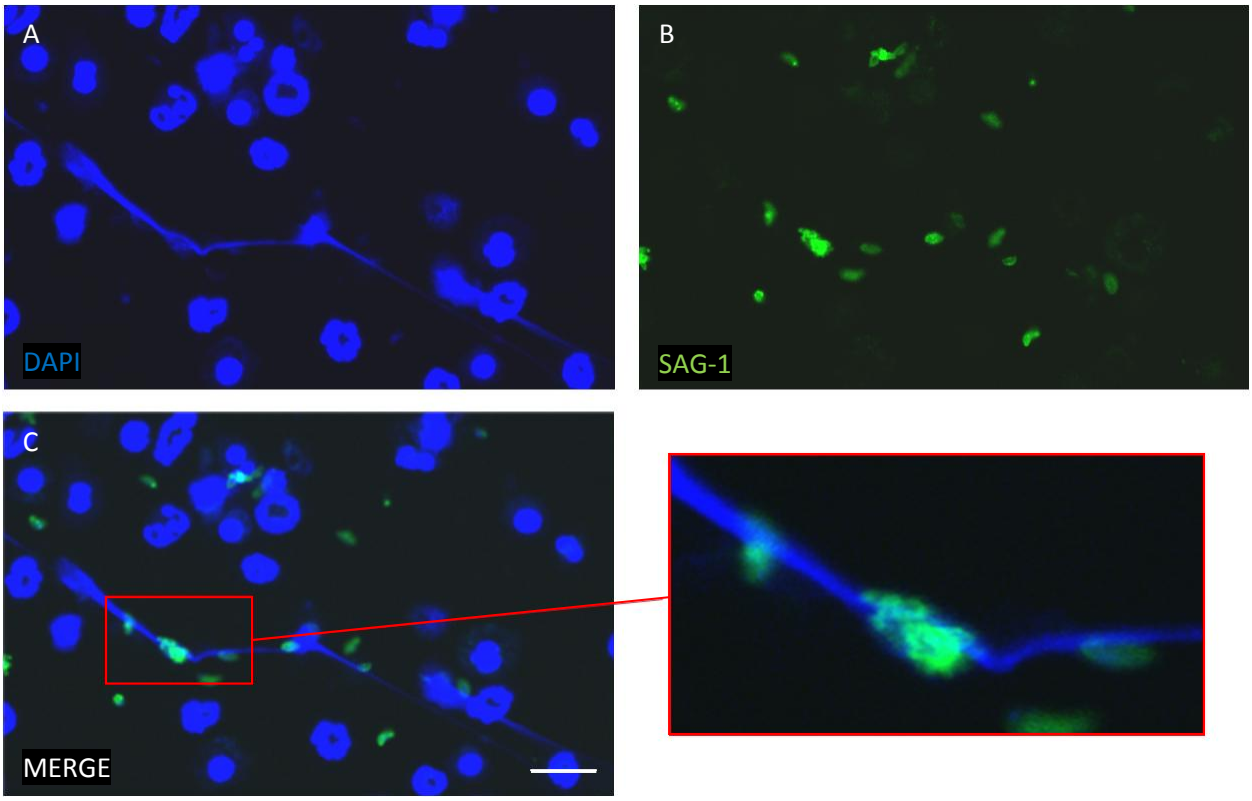
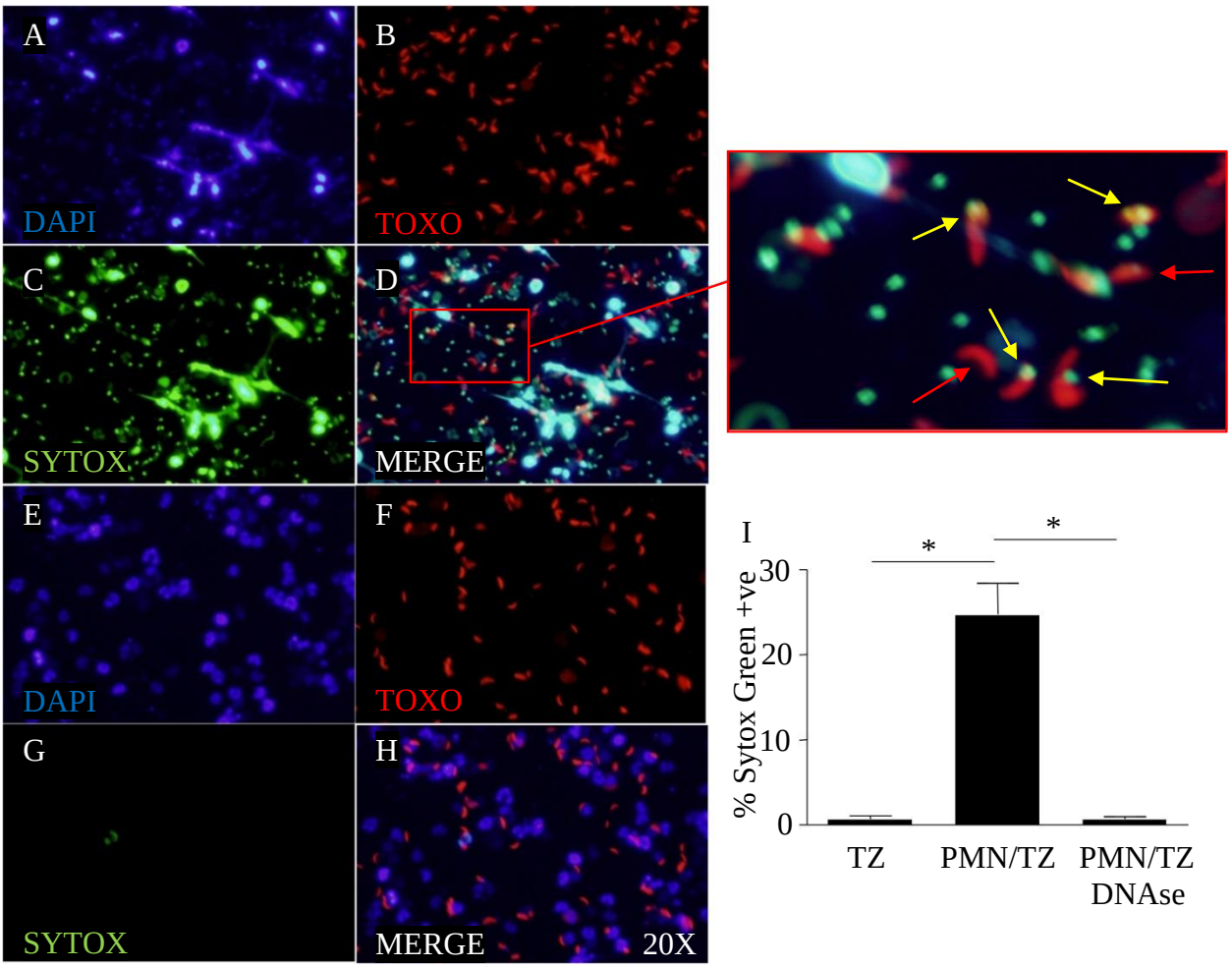


Figure 3.4. NET formation in murine neutrophils does not require invasion. Neutrophils were incubated with *Toxoplasma* (RH strain, MOI 5: 1)) for 4 hrs in the presence of cytochalasin D (1 μM). A, DAPI staining of DNA reveals presence of NETs. B, the same image showing parasite SAG-1 staining. The merged image in C, and the expanded area within the red box reveals SAG-1-positive material entrapped within a NET. The scale bar in C represents 10 μm. This experiment was performed on three independent occasions.

Killing of T. gondii in NETs

We next assessed the viability of parasites entrapped within NETs, since these structures are known to contain anti-microbial components. To address this issue, we made use of Sytox Green, a live-cell exclusion dye that binds to dsDNA within dead cells or parasites, and that would also be expected to bind NETs. These experiments also employed a transgenically modified RH parasite strain that constitutively expresses Red Tomato fluorescent dye. In the presence of cytochalasin D neutrophils generated NETs (Figure 3.5A) during co-incubation with fluorescent tachyzoites (Figure 3.5B). Staining with Sytox Green revealed that many tachyzoites were nonviable (Figure 3.5C and D). In this experiment, less than 1% of tachyzoites stained with this live-cell exclusion dye in the absence of neutrophils, but after co-incubation approximately 25% of parasites were Sytox Green positive (Figure 3.5I). To determine whether parasite death was dependent upon NET formation as opposed to some other mechanism of killing, parallel experiments were performed in the presence of DNase. As expected, inclusion of this nuclease prevented formation of NETs (Figure 3.5E and G). Strikingly, tachyzoites that were present in these cultures (Figure 3.5F) failed to take up Sytox Green (Figure 3.5G and H). In this experiment, less than 1% of parasites took up this dye when DNase was included to prevent NET formation (Figure 3.5I). We conclude that NETs promote extracellular killing of parasites.


A
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% Sytox Green +ve
30
20
10
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TZ
PMN/TZ
PMN/TZ
DNAse

Figure 3.5 (previous page). Parasites are killed in the presence of NETs. In panels A-D, neutrophils were incubated with transgenic RH strain parasites expressing Tomato Red fluorescent protein in the presence of cytochalasin D, and 4 hr later cells were stained with the live-cell exclusion DNA dye Sytox Green. A, DAPI stain; B, Red fluorescence associated with parasites; C, Sytox Green staining. Panel D and the enlarged field show the merged image. Yellow arrows indicate parasites whose nuclei stain with Sytox Green (nonviable) and red arrows indicate tachyzoites that exclude the dye (viable). In parallel (panels E-H), the experiment was carried out in the presence of DNase (1 μg ml^{-1}). Under this condition, NETs failed to form (E and G) and parasites (F) excluded Sytox Green (G and H). Panel I shows quantitation of Sytox Green positive parasites incubated alone (TZ), in the presence of neutrophils (PMN/TZ) and in the presence of neutrophils with DNase (PMN/TZ/DNase). * $p<0.05$. This experiment was performed on three independent occasions.

Human neutrophils release NETs in response to Toxoplasma gondii

Next, we sought to determine whether human neutrophils also responded to *Toxoplasma* with the formation of extracellular traps. To address this question, we first assessed extracellular release of nuclear DNA employing the human promyelocytic leukemia cell line HL-60 that can be induced to differentiate into mature, neutrophil-like myeloid cells when treated with dimethylsulfoxide (30). We determined whether differentiated HL-60 cells responded to parasites by extruding DNA using the PicoGreen DNA assay. As shown in Figure 3.6A, Type II PTG parasites stimulated release of extracellular DNA with kinetics similar to that seen using mouse PMN (Figure 3.2A). Examination of the cells revealed presence of Sytox Green-positive DNA strands similar to that with stimulated mouse neutrophils (Figure 3.6B).

We then sought to determine whether or not freshly isolated human peripheral blood neutrophils also released NETs upon parasite stimulation. Human neutrophils activated with PMA underwent a robust, time-dependent release of extracellular DNA as measured by PicoGreen staining (Figure 3.7A). Cells infected with *Toxoplasma* also underwent similar release of extracellular DNA, and as predicted from the behavior of mouse neutrophils, blocking invasion with cytochalasin D had no effect on DNA release (Figure 3.7A). NET release by human neutrophils was confirmed visually by immunofluorescence assay (Figure 3.7B-E). Cells were incubated with *T. gondii* in the presence of cytochalasin D for 4 hr then fixed and stained for parasites and DNA. Figure 3.7B and C dramatically shows an extensive network of extracellular traps formed in response to *Toxoplasma.* In the merged image (panel D), evidence for entrapment of parasites is clearly visible.

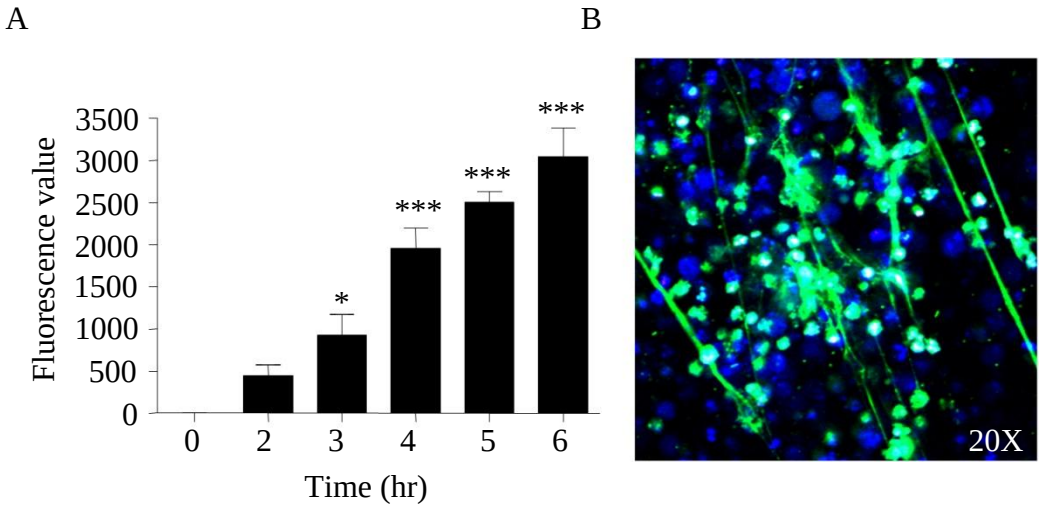


Figure 3.6. Differentiated HL-60 cells form extracellular traps in response to *Toxoplasma*. HL-60 cells were differentiated into neutrophil-like cells with DMSO then subsequently cultured with *T. gondii*. A, Extracellular DNA release over time was measured by PicoGreen binding assay. B, Image collected 6 hr after culture showing PicoGreen positive NET-like structures induced by *Toxoplasma*. The experiments were repeated at least 3 independent times with similar results.

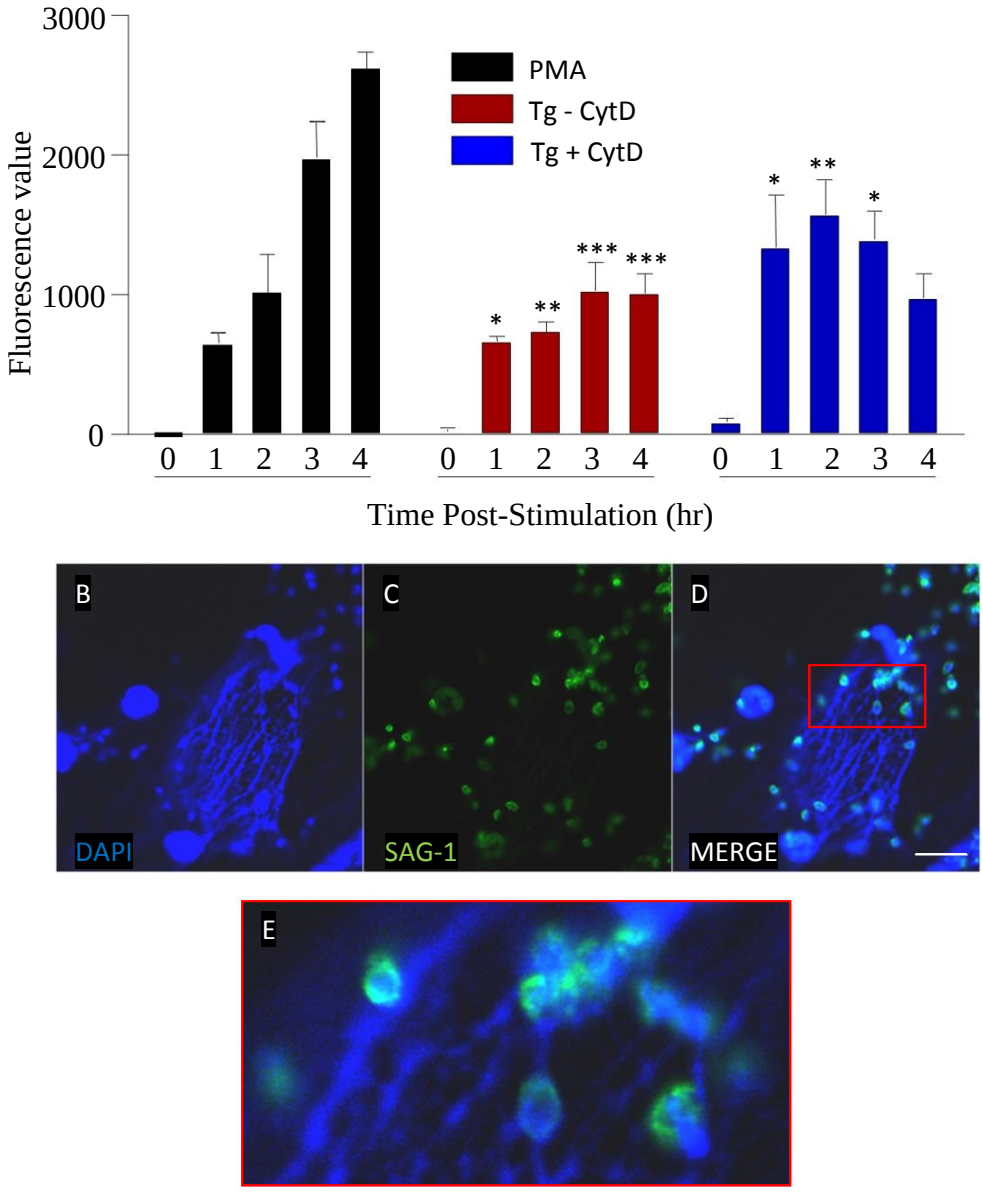


Figure 3.7. Human peripheral blood neutrophils produce NETs in response to *Toxoplasma.* Purified neutrophils were incubated with 60 nM PMA or tachyzoites (MOI 5: 1) with or without cytochalasin D (CytD; 1 μM), and release of DNA was measured over time using PicoGreen fluorescence assay. * $p < 0.05$. B-E, Visual confirmation of NET formation in response to parasites at 4 hr post-incubation as determined by DNA release (B) in the proximity of parasites (C). The merged image in D and the expanded view in E shows extensive parasite entrapment within NETs. Results are representative of at least 3 independent experiments.

Inhibition of ERK1/2 MAPK activation blocks Toxoplasma-induced NET formation

Intracellular control of NET formation is still not well understood, but there is evidence for involvement of a Raf-MEK-ERK signaling pathway (32). Therefore, we sought to determine if *Toxoplasma* activates the ERK1/2 pathway, and whether this signaling kinase is involved in parasite-induced NETosis. Accordingly, we infected human neutrophils with parasites and collected samples for Western blot analysis of ERK1/2 activation. Figure 3.8A demonstrates that *Toxoplasma* triggers ERK1/2 phosphorylation as early as 5 min post-infection and that phosphorylation was sustained for at least 1 hr. Incubating the cells with medium alone did not induce any noticeable phosphorylation of ERK. To determine whether ERK1/2 phosphorylation could be blocked, we used U0126, a well-known chemical inhibitor of MEK1/2 that serves as the upstream MAPK kinase phosphorylating ERK1/2. Cells were treated for 2 hr with U0126 then infected with RH strain parasites in the continued presence of the inhibitor. As expected, the inhibitor completely blocked ERK1/2 phosphorylation triggered by *Toxoplasma* infection (Figure 3.8A). In the same cell lysates, we found high levels of p38 MAPK activation even in the absence of parasites, and phosphorylation of this MAPK was not affected by the inhibitor.

We next asked whether inhibition of ERK1/2 activation affected NETosis triggered by *Toxoplasma*. Neutrophils in the presence of MEK1/2 inhibitor or an equimolar concentration of DMSO were infected with *Toxoplasma* and NET formation was monitored. As expected, NETs were formed in the presence of parasites in medium alone (Figure 3.8B and C). NETosis was decreased when ERK1/2 activation was blocked. Nevertheless, despite the potency of U0126 in blocking ERK activation, NET formation was not completely inhibited, and there was no

significant difference in the presence and absence of inhibitor at later time points (Figure 3.8C). We conclude that optimal NET formation requires ERK1/2 signaling, but that this MAPK is not absolutely needed for the formation of extracellular traps.

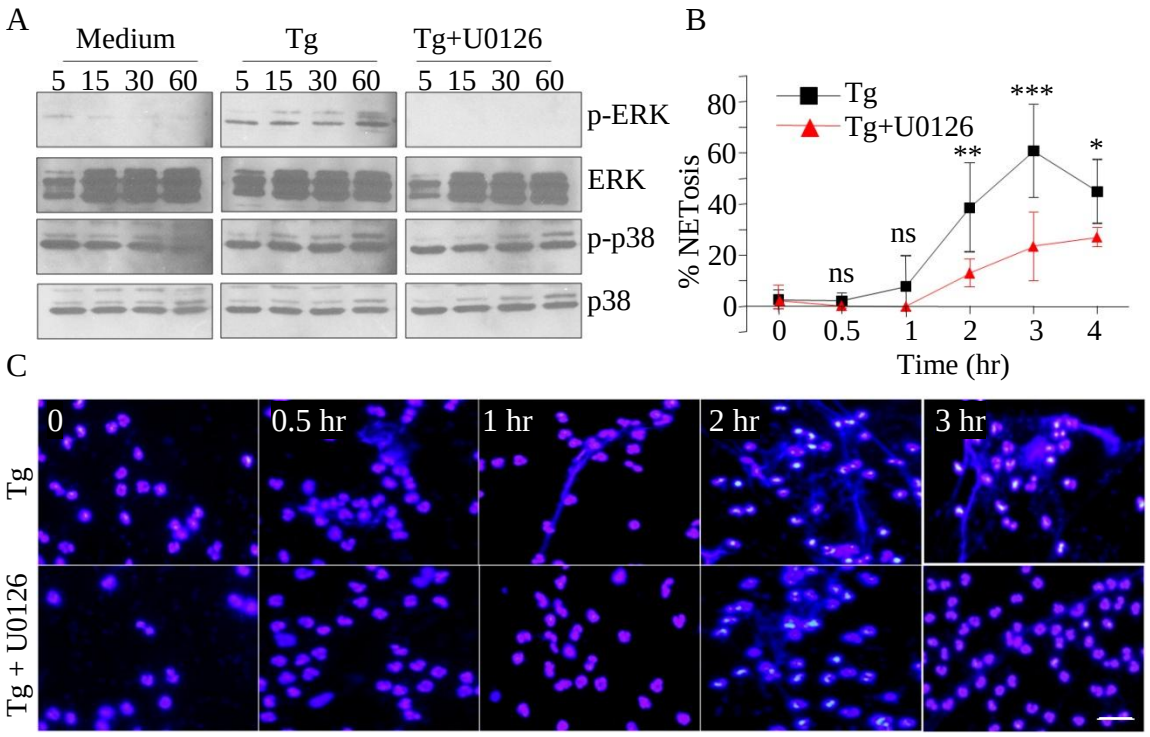
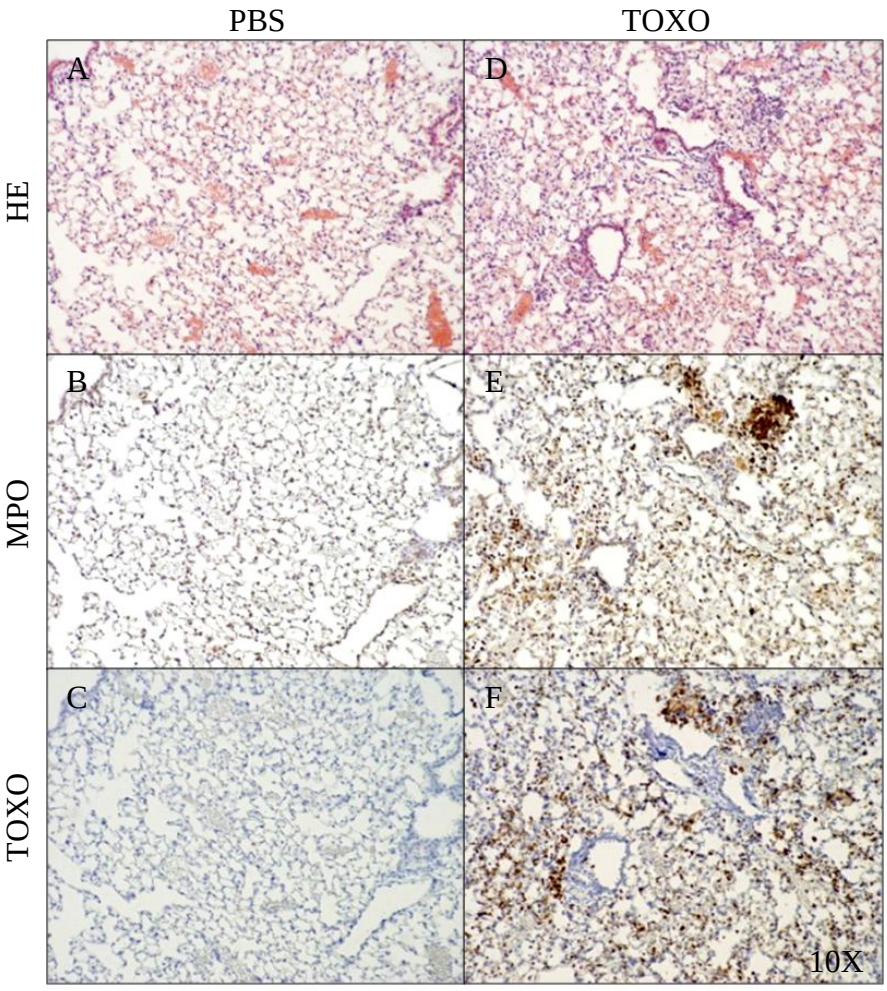


Figure 3.8. Activation of ERK1/2 in response to *Toxoplasma* controls NET extrusion. A, Human peripheral blood neutrophils were incubated in medium alone or with *Toxoplasma* (Tg) alone or with the MEK inhibitor U0126. Western blotting was performed to determine phosphorylation of ERK and p38 MAPK, and blots were stripped and re-probed for total MAPK levels. Panel B shows images over time from DAPI-stained cells incubated with parasites (top panels) or with parasites and ERK inhibitor (bottom panels). Panel C shows NET formation over time by neutrophils either treated or not treated with the MEK1/2 inhibitor using the Picogreen DNA measuring kit. These experiments were repeated 3 times with similar results.

Toxoplasma induces NET formation in vivo

Finally, we asked whether *T. gondii* could elicit NET formation by PMNs recruited at a site of acute inflammation. To address this question, we employed a *Toxoplasma* mouse infection model in which parasites were administered intranasally and lungs were assessed 6 hrs later for parasite colonization, neutrophil recruitment, and NET induction. Lungs from PBS injected mice displayed normal pulmonary architecture with minimal cellular infiltration (Figure 3.9A) with no detectable myeloperoxidase (MPO) positive cells (Figure 3.9B), and no *Toxoplasma* Ab-reactive cells (Figure 3.9C). However, lungs from infected mice lost normal pulmonary architecture and this was associated with massive cellular infiltration (Figure 3.9D). The infiltrating cells were mostly composed of neutrophils, as determined by expression of MPO in serial sections (Figure 3.9E). Large numbers of parasites were also present in the lung tissue (Figure 3.9F). Therefore, in this in vivo model of parasite-induced inflammation, neutrophils and *Toxoplasma* are brought into close proximity in infected tissue. Next, bronchoalveolar lavage fluid (BALF) was collected from the lungs of PBS administered and Toxoplasma infected mice. After a spin-down step to remove cells, supernatants were tested for dsDNA as a measure of NET formation. Samples from *Toxoplasma*-infected mice displayed greatly increased amounts of dsDNA compared to PBS-injected control mice (Figure 3.9G). We conclude that in vivo infection signals recruitment of neutrophils that then release extracellular traps in response to the parasite.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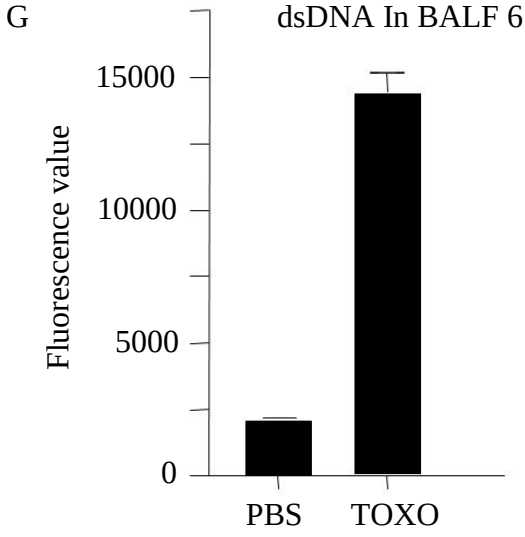
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Fluorescence value
15000
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5000
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PBS
TOXO

Figure 3.9 (previous page). Neutrophils form NETs in vivo during *Toxoplasma* infection. Mice were intranasally injected with PBS as a control (A-C) or intranasally infected with PTG strain parasites (D-F). 6 hr later, lungs were collected, sectioned and serial sections were stained with H&E (A, D), Ab to myeloperoxidase (MPO; B and E), and an anti-*T. gondii* antiserum (TOXO; C and F). In panel G, mice were intranasally administered PBS or tachyzoites, then 6 hr later BALF was collected and dsDNA was measured in cell-free supernatants. These experiments were repeated three times with similar results.

Discussion

Neutrophil extracellular traps have recently emerged as powerful weapons used by PMN to combat microbial pathogens. The results of the present study demonstrate that both mouse and human PMN undergo NET production in response to the protozoan pathogen *T. gondii*. We report that neutrophils respond to *Toxoplasma* infection by releasing extracellular traps and that DNA release is not dependent on active invasion by the parasite or host cell phagocytic function. PMN formed extracellular traps in response to all 3 clonal lineages of *T. gondii*. We also show parasite-induced NET release involves a MEK-ERK pathway. Finally, we found evidence for NET formation during in vivo infection with *Toxoplasma*.

Neutrophil extracellular traps are primarily composed of DNA, histones and other antimicrobial components forming an extracellular mesh that can trap and inactivate pathogens (38). In addition to histones, the antimicrobial components have been shown to include bacterial permeability-increasing protein (BPI), myeloperoxidase, as well as neutrophil elastase (9, 39) that are effective in killing Gram-positive and Gram-negative bacteria (9, 33). It has also been shown that NETs are an important protective mechanism during fungal infection, and that they contain calprotectin, a molecule involved in defense against *Candida albicans* (16, 34). We also obtained evidence for tachyzoite killing that depended upon formation of NETs. At present we do not know what specific components of the NETs mediate parasite killing.

We hypothesize that another major functional consequence for NET formation in the context of *T. gondii* infection may be that they interfere with host cell invasion by ensnaring parasites. As an obligate intracellular protozoan, it is essential for *Toxoplasma* to establish itself within its

intracellular niche so that it can scavenge the host cell for essential nutrients enabling parasite survival and replication. Therefore, any parasite entrapped within a NET will ultimately die as a result of failure to successfully invade a host cell.

The results reported here are amongst the first to show NET formation in response to protozoan parasites. Elsewhere, it has been reported that *Leishmania* triggers NET formation. For the case of *Leishmania amazonensis*, NET entrapment resulted in parasite killing partly dependent upon presence of histones (18). Other studies with *L. donovani* promastigotes showed formation of NETs, but in this case parasites evaded killing in dependence upon expression of lipophosphoglycan (17). There is also evidence for *Plasmodium falciparum*-associated NET formation, and elicitation of anti-nuclear antibodies as a result of DNA release may play a role in pathology in infected children (40). Collectively, these studies contribute to an emerging view that release of extracellular traps by neutrophils is a critical innate defense mechanism that is elicited by a broad range of microbial pathogens.

NETosis is a newly recognized pathway of programmed fatality that enables neutrophils to exert anti-microbial activity even after the cells have died. The process of NETosis appears to involve histone citrullination-dependent chromatin decondensation, disintegration of the nuclear membrane, followed by mixing of nuclear and cytoplasmic effector proteins before eventual release to the extracellular milieu (33, 41-43). The molecular mechanisms underlying this novel type of programmed cell death is an area of intense investigation (44). NETosis has been shown to depend on the generation of reactive oxygen species and a fully functional NADPH complex (45). Neutrophils isolated from patients with chronic granulomatous disease (CGD), who have

defects in NADPH oxidase activity, fail to exhibit NET formation (33, 46). Also, myeloperoxidase and neutrophil elastase have been shown to regulate the formation of neutrophils extracellular traps (47, 48). Recently, Hakkim et al. identified a signaling pathway involved in extracellular trap formation that involves a Raf-MEK-ERK pathway (32). We previously reported that *Toxoplasma* triggers JNK MAPK activation in neutrophils (28), prompting us to examine whether ERK activation could mediate parasite-induced NET extrusion. Indeed, we found evidence for parasite-triggered ERK phosphorylation, and pharmacological inhibition of this cascade blocked the NET response to *T. gondii* infection.

We considered the possibility that NET formation was triggered from within infected cells. However, blocking invasion with cytochalasin D had no effect on formation of NETs. In addition, soluble sonicated parasite lysate could also trigger NET release, although not as efficiently as live parasites (data not shown). Taken together, it seems most likely that production of extracellular traps is mediated by factors released by extracellular tachyzoites. Nevertheless, while cytochalasin D blocks invasion, parasites are still able to attach to the host cell surface and discharge proteins into the host cell (49, 50). It is possible that this phenomenon is involved in stimulating NET extrusion.

The in vivo relevance of NET formation during infection with *Toxoplasma* and other microorganisms is not yet clear. However, we used a mouse intranasal infection model to obtain evidence for a role of NET formation in innate pulmonary defense against *T. gondii*. While this is not a natural route of infection, it is nevertheless well established that parasites disseminate to

the lungs and cause pneumonia in humans and animals (51, 52). Furthermore, MyD88-dependent recruitment of neutrophils to the lamina propria occurs after oral infection with the parasite and there is evidence that neutrophils protect against infection (53-55). Whether protection is a result of interference with invasion by NET entrapment or other neutrophil antimicrobial functions is not known, and may be difficult to determine until more is known about control of NET extrusion. Regardless, our data and those of others demonstrate that NET release is an event that is triggered upon encounter with both prokaryotic and eukaryotic microbial pathogens. How this functions in host defense against infection or possibly might contribute to the disease process will be important issues to address in the future.

Acknowledgments

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CHAPTER 4

Neutrophil Depletion during *Toxoplasma gondii* Infection Leads to Impaired Pulmonary Immunity and Lethal Inflammatory Responses*

Abstract

Considerable confusion surrounds the role of polymorphonuclear leukocytes (PMN) in mouse models of infection. This is because the classical monoclonal antibody (mAb) used to deplete neutrophils (RB6.8C5) recognizes Ly6C/G (Gr-1), which is expressed by plasmacytoid DCs and inflammatory monocytes, in addition to PMNs. Here, we evaluated the importance of neutrophils during *Toxoplasma gondii* infection using the mAb 1A8 that recognizes Ly6G and is therefore neutrophil specific. We determined that neutrophils are required for survival of infected mice in the acute phase of infection. No differences in parasite burden were observed between control and neutrophil-depleted infected mice as determined by PCR and immunohistochemical staining. Histopathological analysis of intestine, liver, brain and spleen failed to reveal any overt differences between neutrophil depleted and control groups. However, histopathology of the lungs from neutrophil-depleted and infected mice showed numerous foci of cellular infiltrates. Flow cytometric analysis and immunohistochemistry revealed that these cells were composed mostly of $CD4^+$ T cells. Interestingly, IL-12 and TNF-α levels were higher in bronchoalveolar lavage fluid of neutrophil depleted mice. In addition, cells from tracheobronchial lymph nodes draining the lungs of neutrophil depleted mice secreted highly elevated levels of IL-12, TNF-α and IFN-γ. Immunophenotyping revealed that $CD4^+$, $CD8^+$ T cells and $CD11b^+$ cells were the source of these cytokines. Dendritic cells taken from neutrophil depleted and infected mice are more activated and present antigen more efficiently. Taken together, these data indicate a role for neutrophils as immunomodulatory cells that influence the outcome of infection with *T. gondii*.

Introduction

Cells of the immune system play important roles in fighting infection and disease. One of the major first-responders to any source of foreign insult are polymorphonuclear leukocytes, better known as neutrophils (1). They are the first cells to reach a site of infection or inflammation in response to inflammatory signals, thus rendering them important in almost all immune responses. Resolution of inflammation usually involves the apoptosis of neutrophils and their engulfment by phagocytes (2).

Toxoplasma gondii is an obligate intracellular parasite that poses minimal risk to immunocompetent individuals but can wreak havoc in immunocompromised hosts (3, 4). In addition to the risk associated with compromised hosts, the parasite can cause birth defects or even abortions in congenital infections (5). The parasite normally induces a T_h1 type immune response, characterized by the production of IL-12 and IFN-γ, which keeps the infection in check and forces the parasite into a dormant cyst state that has the potential of reactivating at later stages in life causing encephalitis (6-9).

Neutropenic mice are extremely susceptible to a wide range of infectious agents. Mice depleted of neutrophils, using antibody to the neutrophil-associated marker Gr-1 fail to control infections with *Aspergillus fumigates, Candida albicans, Chlamydia trochomatis, Legionella pneumophilia, Leishmania infantum, Leishmania major, Toxoplasma gondii* and *Listeria monocytogenes* among other pathogens (10-25). Mice depleted of Gr-1^+ cells and infected with *T. gondii* succumb to the parasite within ten days postinfection with a highly dysregulated cytokine profile. This

dependency on Gr-1$^+$ cells for survival is critical in the first few days of infection, since mice depleted of Gr-1$^+$ cells starting at 6 days postinfection still survive acute infection. In addition, these Gr-1$^+$ depleted mice exhibit increased parasite burdens with exacerbated lesions in multiple organs (24). In the context of a *Toxoplasma* infection, neutrophils produce a variety of chemokines and cytokines that can influence immune responses. Of particular importance is the production of IL-12, TNF-α, MIP-1β, MIP1-α in human neutrophils, and MyD88-dependent IL-12 and MCP-1 in mouse neutrophils in response to parasite stimulation (26, 27). Also of interest is the fact that during a *Toxoplasma* infection, neutrophils with prestored IL-12 are recruited to the site of infection (28).

Rendering mice neutropenic using antibody-mediated depletions with the anti-Gr-1 antibody has been the standard protocol to achieve depletions (29). However, recent evidence suggests that the surface marker Gr-1 is also expressed on plasmacytoid dendritic cells in addition to CCR2$^+$ inflammatory monocytes (30, 31). A more recent study makes use of a recently available monoclonal antibody that targets the neutrophil-specific Ly6G surface molecule (32). This antibody is neutrophil specific and should not deplete other Gr-1$^+$ cell populations.

Here we show that we can effectively and efficiently deplete mice of neutrophils using the 1A8, anti-Ly6G depleting antibody. We also show that mice depleted of neutrophils succumb to infection within 10 days. Depletion of neutrophils however had no statistically significant effects on parasite burden in both lymphoid and non-lymphoid tissues. Neutrophil depletion, followed by infection, led to an increase in the cytokine levels of IL-12, TNF-α, IL-10, and IFN-γ

observed in bronchoalveolar lavage fluid (BALF) and tracheobronchial lymph node (TLN) culture supernatants. This overproduction of cytokines was due mostly to an increased and activated population of $CD4^+$ T cells and also due to an increased production of cytokines from $CD8^+$ T cells and $CD11b^+$ cells in neutrophil depleted mice. We also observed that $CD11c^+$ cells from neutrophil depleted and infected mice were more activated and led to an increased T cell proliferative response when pulsed with OVA and incubated with OT-II OVA specific $CD4^+$ T cells.

Materials and Methods

Mice

Female C57BL/6 mice were purchased from Taconic Farms (Germantown, NY, USA) and C57BL/6-Tg(TcraTcrb)425Cbn/J (OT-II) breeders were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and used at 6-8 wk of age. All mice were maintained in the Transgenic Mouse Core Facility at the Cornell University College of Veterinary Medicine, which is accredited by the American Association for Accreditation of Laboratory Animal Care.

Depletions, parasites and infections

Mice were rendered neutropenic by injections with anti-1A8 (anti-Ly6G, Bioxcell, West Lebanon, NH, USA) and anti-Gr-1 (RB6C6.8C5 hybridoma originally provided by R. L. Coffman, Dynavax Technologies Corporation, Berkeley, CA, USA) monoclonal antibodies. Depletion of IL-12 in vivo was achieved by injecting mice with anti-IL12 antibody

(hybridoma originally provided by G. Trinchieri, NCI, Frederick, MD, USA). Mice were injected with 1 mg of anti-1A8, 200 µg of anti-RB6 or 200 µg of anti-IL-12 antibodies every 48 hrs starting at day -2 prior to infection and up to day 8 post-infection. Control animals were injected with 1 mg of Rat IgG antibody.

ME49 bradyzoite cysts were maintained in Swiss Webster mice as previously described (33). Brains of chronically infected mice were collected, brain homogenate obtained and cysts counted. Mice were infected orally with 20 cysts in a 250 µl volume using an oral gavage needle.

Soluble tachyzoite antigen (STAg) was prepared as previously described (33).

Histology and Tissue collection

Mice were euthanized at days 8 or 9 post-infection and dissected for tissue collection. Gut, livers, lungs, brains and spleens were collected and fixed in 10% (wt/vol) buffered formaldehyde. Samples were then progressively dehydrated in ethanol, cleared with xylene, and embedded in paraffin for further staining with hematoxylin and eosin or anti-CD3. Samples were also embedded in OCT solution for cutting on a cryotome.

BALF collection and Lung digestion

Bronchoalveolar lavage fluid (BALF) and lungs were collected from day 8 antibody injected and infected mice. Briefly, after the mice were euthanized, a small tracheal incision was made and

lung was washed using PBS with a 200 µl loading tip for a total of 5 washes. The resulting fluid was separated by centrifugation into cells for flow cytometric analysis and supernatant for cytokine measurement analysis. Lungs of day 8 infected mice were collected and mechanically sliced using a razor blade into 1 mm thick sections. Resulting sliced sections were further enzymatically digested using both Liberase at 0.2 mg ml^{-1} (Roche Diagnostics, Mannheim, Germany) and Collagenase at 5 mg ml^{-1} (Sigma-Aldrich, St Louis, MO, USA) for 1 hr at 37°C while gently rocking. Cells were then washed 3X in Hanks Balanced Salt Solution, filtered using a 70 micron filter (BD Falcon, Franklin Lakes, NJ, USA) and subjected to multi-colored flow cytometric analysis.

STAg restimulation and cytokine measurement

Mesenteric lymph nodes (MLN), spleens (SPL) and tracheobronchial lymph nodes (TLN) were collected from day 8 mice and single cell suspensions obtained by gently mashing. Cells were filtered using a 70 micron filter and red blood cells lysed using ACK lysis buffer (Gibco, Grand Island, NY, USA). Cells were seeded onto a 96 well plate (BD Falcon, Franklin Lakes, NJ, USA) at a concentration of 2 x 10^6 cells per well in a 100 µl volume in complete DMEM (cDMEM) consisting of 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 30 mM HEPES, (all purchased from Invitrogen Life Technologies, Carlsbad, CA, USA), 10% bovine growth serum (Hyclone, Logan, UT, USA) and 0.05 mM β-mercaptoethanol. STAg was added in a 100 µl volume and cell-free supernatants were collected after 24 hrs in culture. IL-12p40 was measured in cell-free supernatants as previously described

(33). IL-10, TNF-α, IFN-γ and IL-17 were measured in cell-free supernatants according to the manufacturer's instructions (eBioscience, San Diego, CA, USA).

Toxoplasma burden measurements

DNA extraction from tissue samples, including the gut, brain, liver, lungs and spleen, was performed using DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. Briefly, after 20 mg of tissue samples were digested in lysis buffer with Proteinase K at 55°C, DNA was extracted, washed, and eluted in 200 μl of elution buffer.

Real-time PCR was performed targeting the highly conserved 35-fold-repetitive B1 gene in *T. gondii* (34). A 25 μl reaction mixture was prepared using 2X Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with 0.3 μM of forward primer (5'-GGA GGA CTG GCA ACC TGG TGT CG-3') and reverse primer (5'-TTG TTT CAC CCG GAC CGT TTA GCA G-3') (35). Real-time PCR was carried out in an Applied Biosystem 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA) with the following thermal cycling conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All PCR amplifications were subjected to dissociation analysis to confirm the specificity of amplification. Quantification of the parasites was accomplished by using a standard curve constructed with a series of known quantity of the RH strain of *T. gondii* over a range of 6 logs corresponding to 10^5 to 1 parasite per reaction. Uninfected tissues were used as negative controls and distilled sterile water used as no template controls.

Flow cytometric analysis and intracellular staining

Single-cell suspensions were incubated for 30 min at 4°C with FACS buffer (PBS, 1% bovine growth serum and 0.01% NaN_3) containing 10% normal mouse serum to block Fc receptor binding. Samples were then centrifuged and cells resuspended in FACS buffer containing fluorochrome-conjugated antibodies for 30 min at 4°C. Antibodies used in this study were: anti-Gr-1 FITC (BD Biosciences, San Jose, CA, USA), PE-conjugated anti-Ly6G (BD Biosciences), PE-conjugated anti-CD62L, CD25, CD11b, CD11c, F4/80, NK1.1 and CD69 (BD Biosciences), allophycocyanin-conjugated anti-CD4 and CD8 (BD Biosciences), PE-conjugated anti-IFN-γ (eBioscience, San Diego, CA, USA), and PE-conjugated anti-IL-10 (eBioscience). Cells were then washed and resuspended in FACS buffer and collected on a BD FACSCalibur flow cytometer. Data analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA). For intracellular staining, after cells were surface stained for membrane antigens, they were permeabilized and stained for intracellular cytokines.

OT-II T cell proliferation studies

To obtain DC, lungs from day 8 infected mice were excised and single-cell suspensions were obtained as described above. Cells were centrifuged at 300 × *g* for 10 min at 4°C. The pellet was resuspended in MACS buffer (PBS, 0.5% BSA and 2 nM EDTA) and MACS anti-CD11c magnetic beads (Miltenyi Biotec, Auburn, CA, USA) were added. The sample was mixed and incubated for 15 min at 4°C. Cells were washed using MACS buffer and centrifuged at 300 × *g* for 10 min. The cell pellet was resuspended in MACS buffer and CD11c-positive and negative fractions were separated using an AutoMACS Separator. The same protocol was used to

obtain $CD4^+$ T cells starting with spleens from OT-II mice and using anti-CD4 magnetic beads. $CD4^+$ T cells were stained with 5-(and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, cells were resuspended in PBS with 0.1% BSA and CFSE stock solution was used to yield a final concentration of 10 μM. Cells were incubated at 37°C for 10 min. The staining was quenched by adding five volumes of ice-cold cDMEM followed by incubation on ice for 5 min. CFSE-stained cells were further washed for a total of three washes using cDMEM. DC were pulsed with OVA 323-339 peptide (2μg ml^{-1}) and T cells were incubated together at a ratio of 1:10 for 4 days before assayed for CFSE peak dilutions.

Immunofluorescence microscopy

OCT embedded lungs were sliced on a cryotome to a thickness of 8 μm then collected on charged slides, acetone fixed, and frozen at -80°C until ready to use. After slides were thawed to room temperature, samples were outlined with an Aqua Hold Pap Pen (Fisher Scientific, Pittsburgh, PA, USA) then blocked for 30 min at room temperature using 2X casein (Vector Labs, Burlingame, CA, USA) in PBS containing normal mouse serum (JacksonImmunoResearch, West Grove, PA, USA). Anti-CD4 FITC conjugated antibody (eBioscience, San Diego, CA, USA) was added in block solution for 2 hrs at room temperature in a dark and humid chamber. After the antibody incubation, slides were washed twice in PBS then mounted with ProLong Antifade containing 4′,5-diamidino-2-phenylindoleI (Molecular Probes). Images were collected with an Olympus BX51 fluorescence microscope equipped with a DP 70 camera using Olympus DP controller software and Olympus DP manager software.

Statistical analyses

Statistical analyses were performed using the Prism Software. Unpaired, two-tailed *t*-tests were used to determine statistical significance. *P* values <0.05 were considered significant.

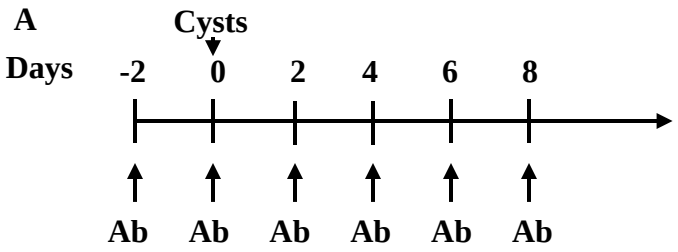
Results

Neutrophil-depleted mice succumb to Toxoplasma infection

Considerable confusion surrounds the role of antibodies in the depletion of neutrophils. Depletion of neutrophils during a *Toxoplasma* infection has routinely been performed using an anti-Gr-1 (RB6.8C5) antibody (25, 36). However, it has recently been shown that other cells can also express Gr-1 molecules on their surface, namely inflammatory monocytes and plasmacytoid dendritic cells (30, 31). 1A8 antibody has been developed as a neutrophil specific antibody that recognizes the Ly6G surface molecule specifically found on neutrophils. Figure 4.1A shows a schematic representation of our depleting protocol, employing anti-Ly6C/G, anti-Ly6G and control IgG antibodies. Mice were treated with antibodies and infected with 20 cysts of ME49 orally, then on day 4 postinfection we looked at the percentage of cells staining positive for neutrophil markers in both spleens (SPL) and mesenteric lymph nodes (MLN). Panels B-E show surface staining of single cell suspension from the spleen and mesenteric lymph nodes of depleted and non-depleted infected mice. The double positive, $RB6^{+}/1A8^{+}$, neutrophil population is absent in the SPL and MLN of 1A8 treated mice (C and E), but still present in the SPL and MLN IgG treated mice (B and D), confirming depletion in vivo. To confirm depletions systemically, we also stained for elastase, a neutrophil marker, in tissues collected from control and neutrophil depleted animals. Figure 4.1F and G shows elastase staining in the gut of day 8

infected mice in both IgG and 1A8 treated mice. Neutrophil treated mice had no positive cells for elastase (Figure 4.1G), whereas elastase positive cells were visible in non-depleted mice (Figure 4.1F, arrows). Depletion of mouse neutrophils was also confirmed by performing blood smears using peripheral blood collected from control and neutrophil depleted mice (Data not shown).

We then asked whether neutrophil depleted mice succumbed to *Toxoplasma* infection. As shown in Figure 4.2A, in vivo depletion of neutrophils with the 1A8 antibody (dotted line) renders the mice more susceptible to infection with 100% mortality by day 10 postinfection. Mice treated with IgG antibody (solid line) survived past day 30 postinfection underscoring the importance of neutrophils in the early control of *Toxoplasma* infection. RB6.8C5 (dashed line) treated mice also succumbed to infection, albeit with different kinetics, due perhaps to a role that inflammatory monocytes can play. Using such a high dose of depleting antibody raised concerns that the mice might be undergoing organ failure as a result of the high antibody dose and not the infection. To address this question, we treated mice with both IgG and 1A8 antibodies at a dose of 1 mg but did not infect with *Toxoplasma*. As can be seen in Figure 4.2B, both groups survived antibody injections ruling out any role for the dosage of antibody on mouse mortality.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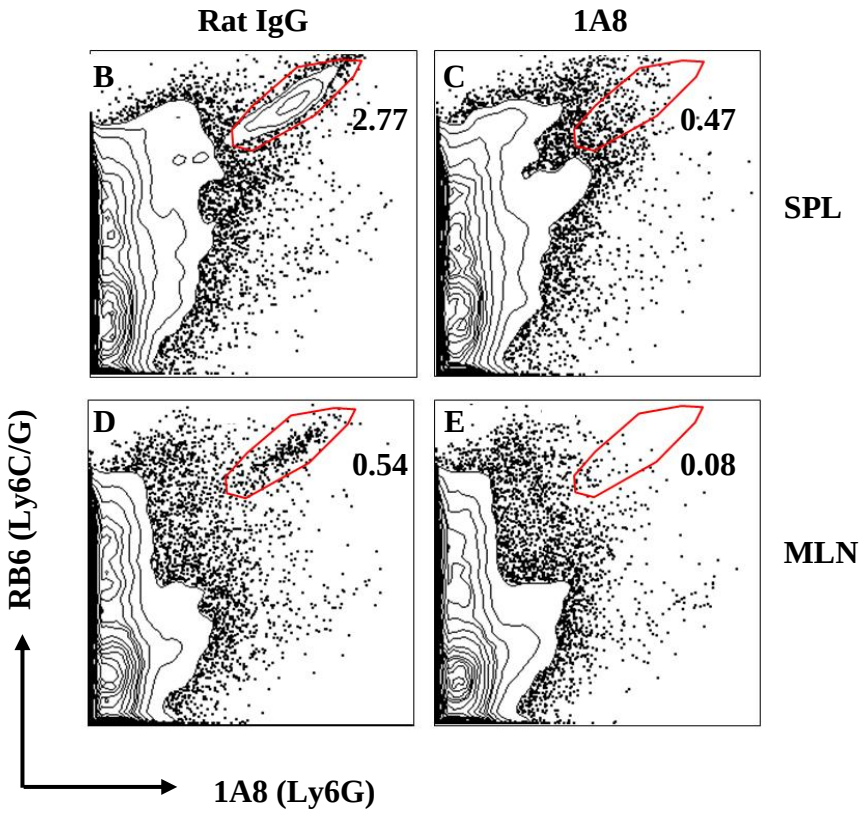
Rat IgG
1A8
B
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0.47
SPL
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0.54
E
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MLN
RB6 (Ly6C/G)
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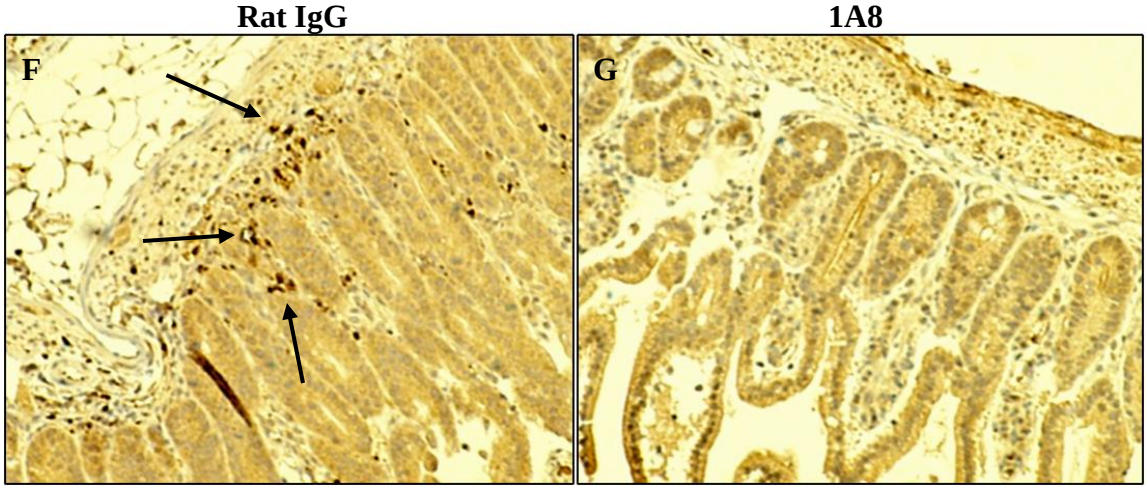

Rat IgG
1A8
F
G

Figure 4.1 (previous page). 1A8 treatment completely depleted neutrophils in vivo. Mice were depleted with 1 mg dose of anti-Ly6G, 200 µg dose of anti-Ly6C/G, 200 µg dose of anti-IL-12 and injected with 1 mg dose of control rat IgG. A, Injections were administered 48 hrs prior to infection with 20 cysts of ME49, and then booster injections were administered every 48 hrs thereafter. B, presence of neutrophils ($RB6^+/1A8^+$) in the spleens of non-depleted infected mice. C, confirmed depletion of neutrophils in the spleens of depleted infected mice. D, presence of neutrophils ($RB6^+/1A8^+$) in the mesenteric lymph nodes of non-depleted infected mice. E, confirmed depletion of neutrophils in the mesenteric lymph nodes of depleted infected mice. All samples shown in B-E are from day 4 postinfection. F, presence of elastase positive cells in the gut of non-depleted infected mice. G, lack of elastase positive staining in the gut of depleted infected mice. Experiments were repeated at least 3 times with similar results.

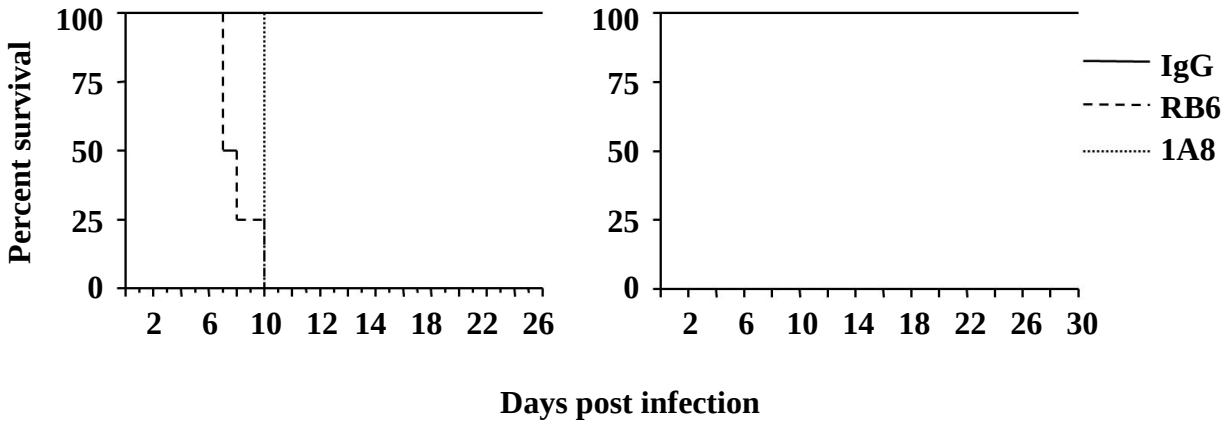
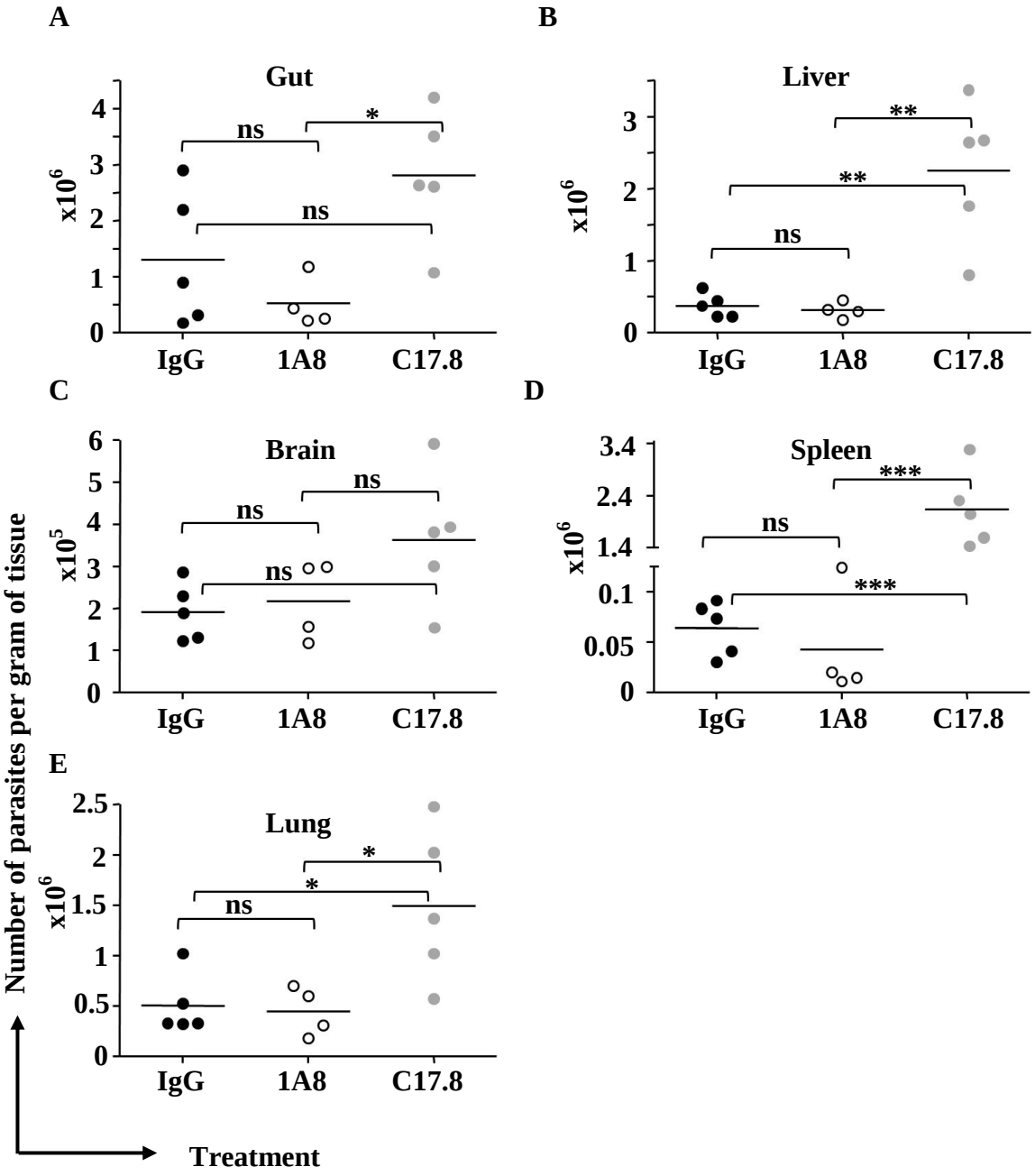


Figure 4.2. Mice depleted of neutrophils in vivo are more susceptible to *Toxoplasma* infection. A, survival curves of neutrophil-depleted (dotted line), antibody-control treated (solid line), or RB6 treated (dashed line) and infected mice. B, survival curves of neutrophil-depleted and antibody-control treated and non-infected mice. Four mice were used for each treatment group. Experiments were performed at least 3 times with similar results.

Neutrophil-depleted and control-injected mice have comparable parasite burdens

We then wondered as to whether neutrophil-depleted mice had a deficit in their capacity to control the replication of the parasite, resulting in mortality. We collected tissues at day 8 postinfection from IgG and 1A8 treated and infected mice. We also treated mice with anti-IL-12 antibody to deplete IL-12, as it has been shown to be important for control of parasite replication in vivo (37). As can be seen in Figure 4.3, there were no significant difference between control (IgG, black circles) or neutrophil-depleted (1A8, white circles) and infected mice in gut (A), liver (B), brain (C), spleen (D) and lung tissues (E). However, in all tissues tested, there were significantly higher numbers of parasites in the IL-12 depleted mice (IL-12, grey circles).


A
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Number of parasites per gram of tissue
Treatment

Figure 4.3 (previous page). Neutrophil-depleted and control-treated and infected mice have equivalent parasite burden in gut, liver, brain, spleen and lung. DNA isolated from tissues was subjected to real-time PCR targeting the highly conserved 35-fold-repetitive B1 gene in *T. gondii*. Samples were collected from mice treated with anti-Ly6G, anti-IL12 and control IgG antibodies and infected with 20 cysts of ME49. No significant differences were observed between the control and neutrophil-depleted groups, however, significant differences were observed between mice depleted of IL-12 and all other groups. All data shows toxoplasma burden per gram of tissue in A, gut, B, liver, C, brain, D, spleen and E, lungs. ns means not significant, * is a p value in the range of 0.01 to 0.05, ** 0.001 to 0.01 and *** is a p-value of< 0.001. Experiments were repeated at least 3 times with similar results.

Neutropenic infected mice have abnormal lung pathology

To examine why mice were susceptible to infection, we collected tissues from all major organs of depleted and infected mice as well as non-depleted and infected mice immediately prior to death, usually around day 8. Figure 4.4 shows representative images from Hematoxylin & Eosin (H&E) stains for gut (A and B), spleen (C and D), liver (E and F) and brain (G and H). At these sites, there were no differences between control (A, C, E and G) and depleted (B, D, F and H). Differences however were observed in the lungs of neutrophil-depleted and infected mice and representative images are shown in Figure 4.5. As can be seen in Figure 4.5B, lungs from neutrophil-depleted and infected mice show increased pulmonary congestion and an infiltration of cells at various foci (red circles), when compared to control-treated and infected mice (Figure 4.5A).

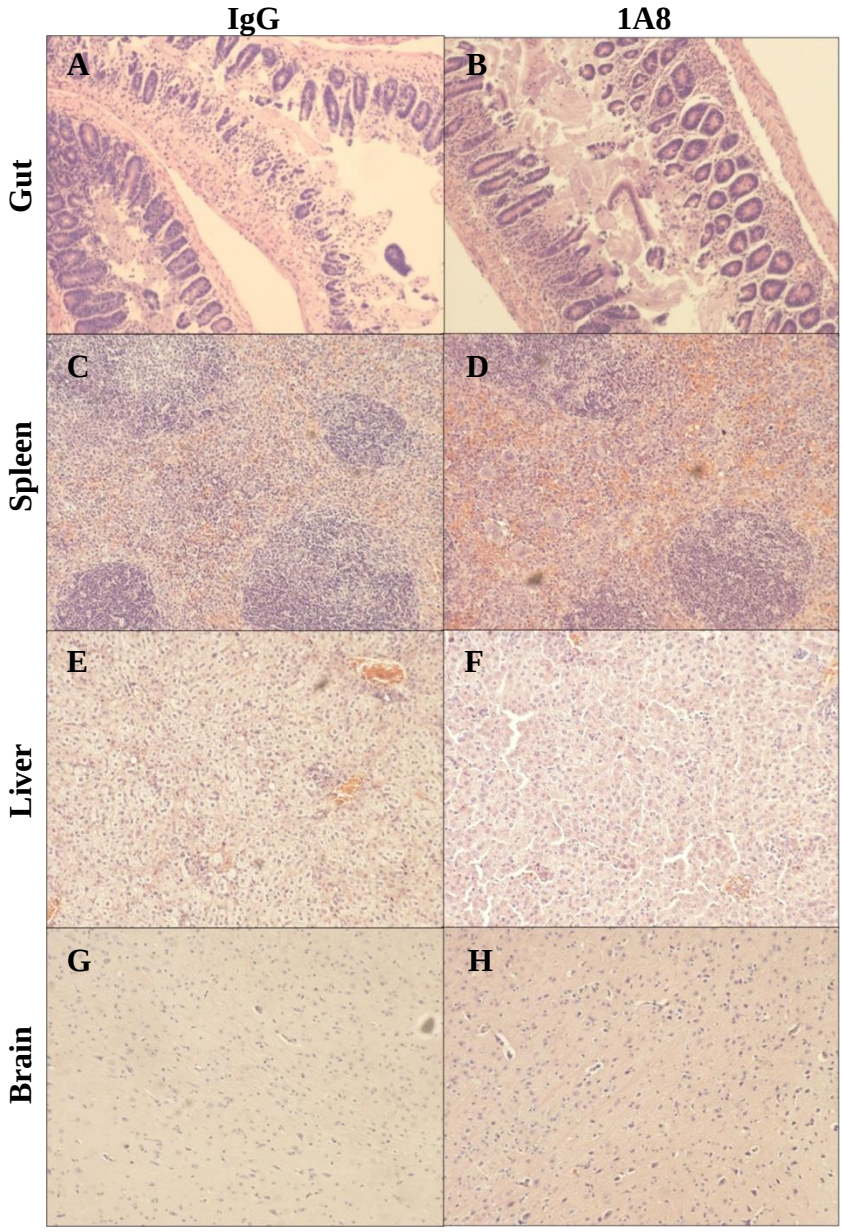
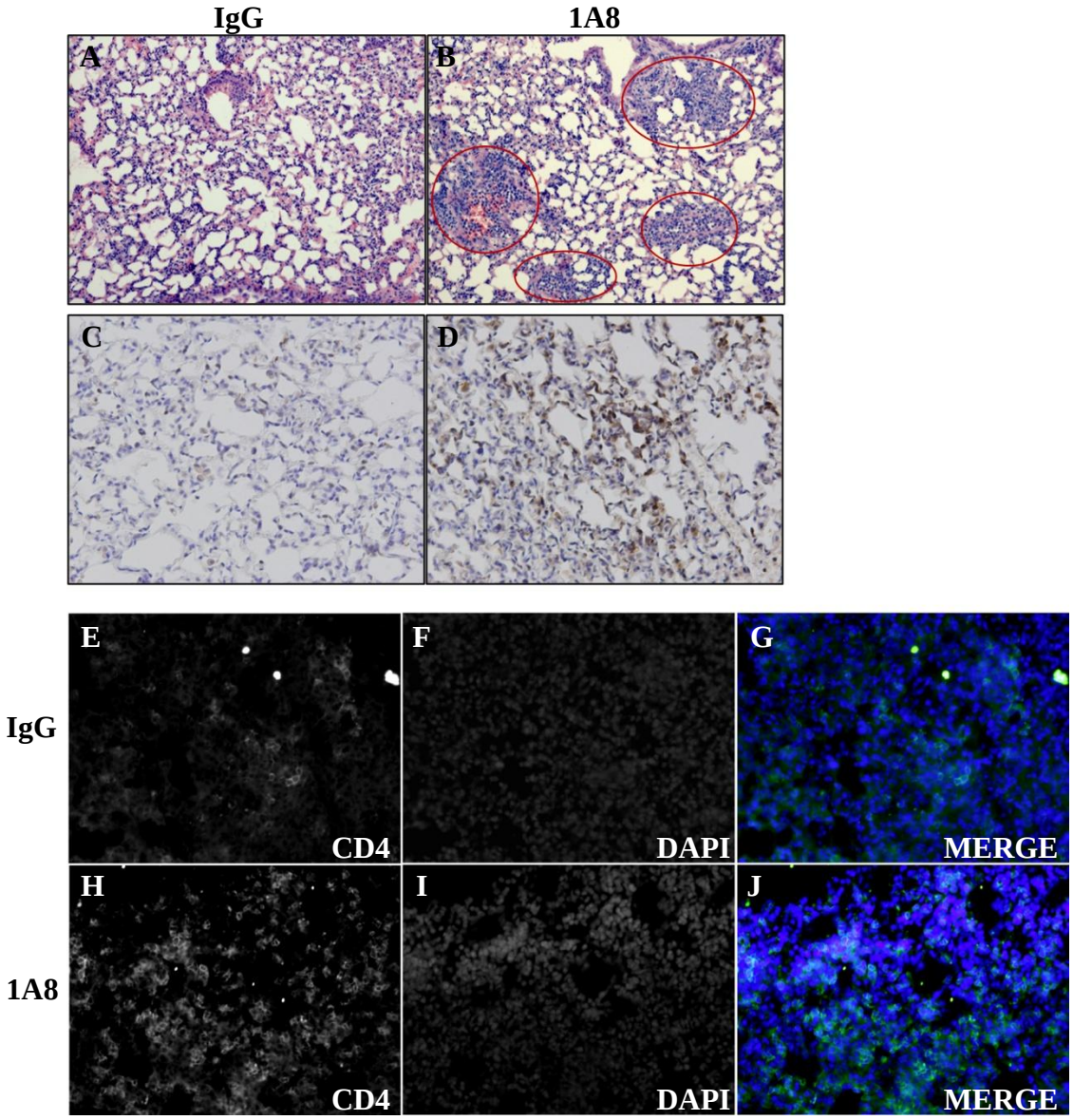


Figure 4.4. Neutrophil-depleted mice exhibit no differences in lesions in both lymphoid and non-lymphoid tissues. Tissues from gut (A and B), spleen (C and D), liver (E and F) and brain (G and H) were collected from control treated (A, C, E and G) and neutrophil-depleted (B, D, F and H) animals at day 8 postinfection with 20 cysts of ME49. Samples were fixed according to standard lab protocols and stained for hematoxylin and eosin. Experiments were repeated at least 3 times with similar results.

Cellular infiltrates in neutrophil-depleted lungs are mostly T cells

We speculated as to the composition of the cellular infiltrates in Figure 4.5B, and performed flow cytometry to determine their composition. Immunohistochemical analysis showed that these cells were mostly $CD3^+$ cells indicating that they were T cells (Figure 4.5D). Further analysis showed that these cells were mostly $CD4^+$ T cells (Figure 4.5H). To further characterize the cellular infiltrates, we collected bronchoalveolar lavage fluid from control and neutrophil-depleted mice at day 8 postinfection and subjected the cells to flow cytometric analysis. As can be seen from Figure 4.6A, neutrophil-depleted mice (white bars) had a much higher percentage of $CD4^+$ T cells when compared to control treated and infected mice (black bars). We also performed analysis on lung digests from control and neutrophil-depleted and infected mice. Lungs from mice were mechanically and enzymatically digested to obtain single-cell suspensions. Resulting cell populations were subjected to flow cytometric analysis. Figure 4.6B shows a modest increase in the percentage of $CD4^+$ T cells in lung digests from neutrophil depleted mice. Lungs of these mice also have slightly elevated percentage of macrophages and B cells evidenced by increased staining for F4/80 and B220 respectively.


IgG
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D
IgG
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DAPI
MERGE
1A8
H
I
J
CD4
DAPI
MERGE

Figure 4.5 (previous page). Lungs of neutrophil-depleted mice have infiltrating cellular foci. Mice were treated with control rat IgG antibody or neutrophil depleting antibody and infected with 20 cysts of ME49. At day 8 postinfection, lungs were collected, fixed, paraffin embedded and stained for H and E and CD3. OCT embedded lungs were stained for CD4. A, Lungs from IgG treated mice have normal pulmonary architecture. B, Lungs from 1A8 treated mice are congested with various cellular foci spread throughout (red circles). C, Control treated mice have minimal CD3 positive cells. D, Lungs from neutrophil depleted mice have higher numbers of cells positive for CD3. E, F and G, CD4, DAPI and merged images for lungs from control treated mice. H, I and J, higher numbers of $CD4^+$ T cells in the lungs of neutrophil depleted mice. All experiments were repeated at least three times with similar results.

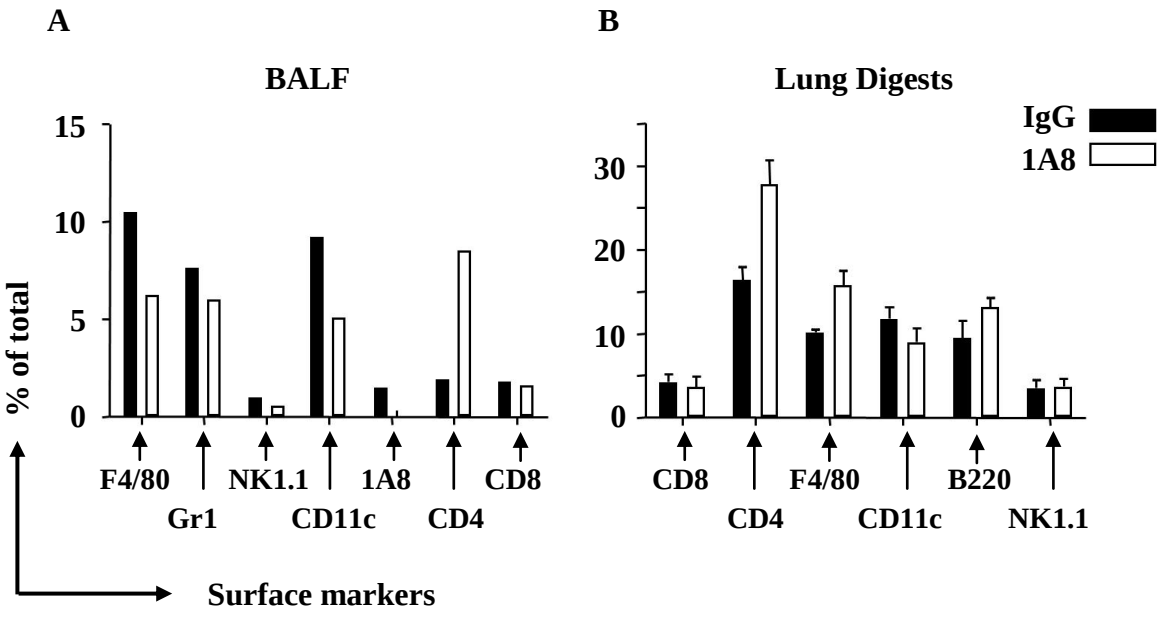

Figure 4.6. Neutropenic mice have higher percentage of CD4$^+$ T cells. Control treated and neutrophil-depleted and infected mice were euthanized on day postinfection and BALF and lungs collected for flow cytometric analysis for cellular composition. Macrophages were stained with F4/80, natural killer cells with NK1.1, dendritic cells with CD11c, T cells with CD4 and CD8, neutrophils with 1A8 and Gr-1. A, BALF of neutrophil-depleted mice have almost a 3 fold increase in the percentage of T cells staining positive for the CD4 marker. B, Increase in percentage of CD4$^+$ T cells in lung digests from neutrophil depleted mice. Experiments were performed with 4 mice per group and repeated at least 3 times with similar results.

Neutrophil depleted mice have impaired pulmonary cytokine profiles

Given that no major difference in parasites replication was observed, we looked at whether control and depleted mice had different cytokine profiles. We collected cell-free supernatants from BALF and subjected the samples to cytokine measurements by ELISA. Supernatants from neutrophil-depleted mice had elevated levels of IL-12 and TNF-α when compared to control treated mice (Figure 4.7). To further characterize the recall immune response, we performed restimulation assays with soluble tachyzoite antigen (STAg) on single-cell suspensions isolated from spleens (SPL), mesenteric lymph nodes (MLN) and tracheobronchial lymph nodes (TLN). Figure 4.8 shows ELISA data from cell-free supernatants obtained from STAg restimulations for 24 hrs ex vivo. All cultures from neutrophil depleted mice (white bars) in TLN cultures had higher levels of cytokines when compared to control treated mice (black bars) in every cytokine tested. We observed higher levels of IL-12 (A), IL-10 (B), TNF-α (C) and IFN-γ (D). Interestingly, this difference is localized to the lungs since restimulation assays with samples collected from spleens and mesenteric lymph nodes had no differences between control and depleted groups.

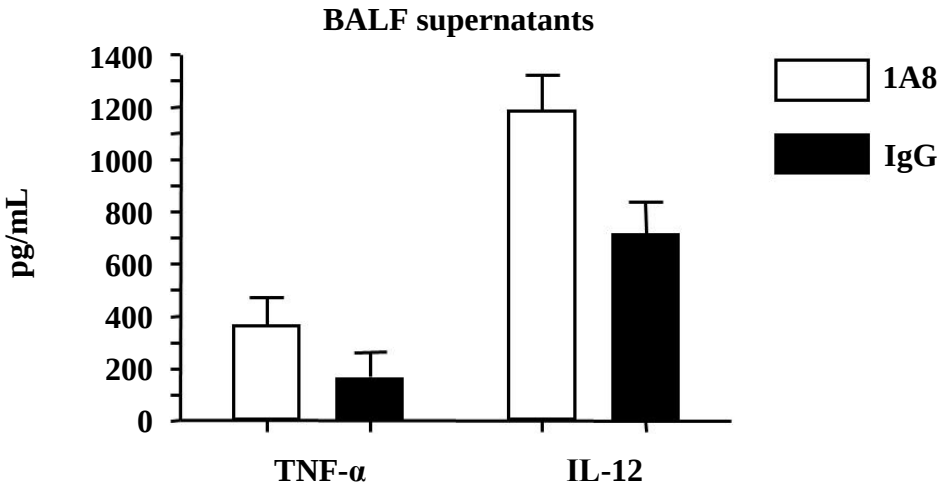

Figure 4.7. Levels of IL-12 and TNF-α are increased in BALF supernatants of neutrophil-depleted mice. BALF collected from control-treated or neutrophil-depleted and infected mice was separated by centrifugation into cells and cell-free supernatants. Supernatants were then tested by ELISA for cytokine levels. Neutrophil-depleted mice have higher levels of both IL-12 and TNF-α when compared to control-treated mice (Empty bars vs solid bars). Experiments were repeated at least twice with similar results.

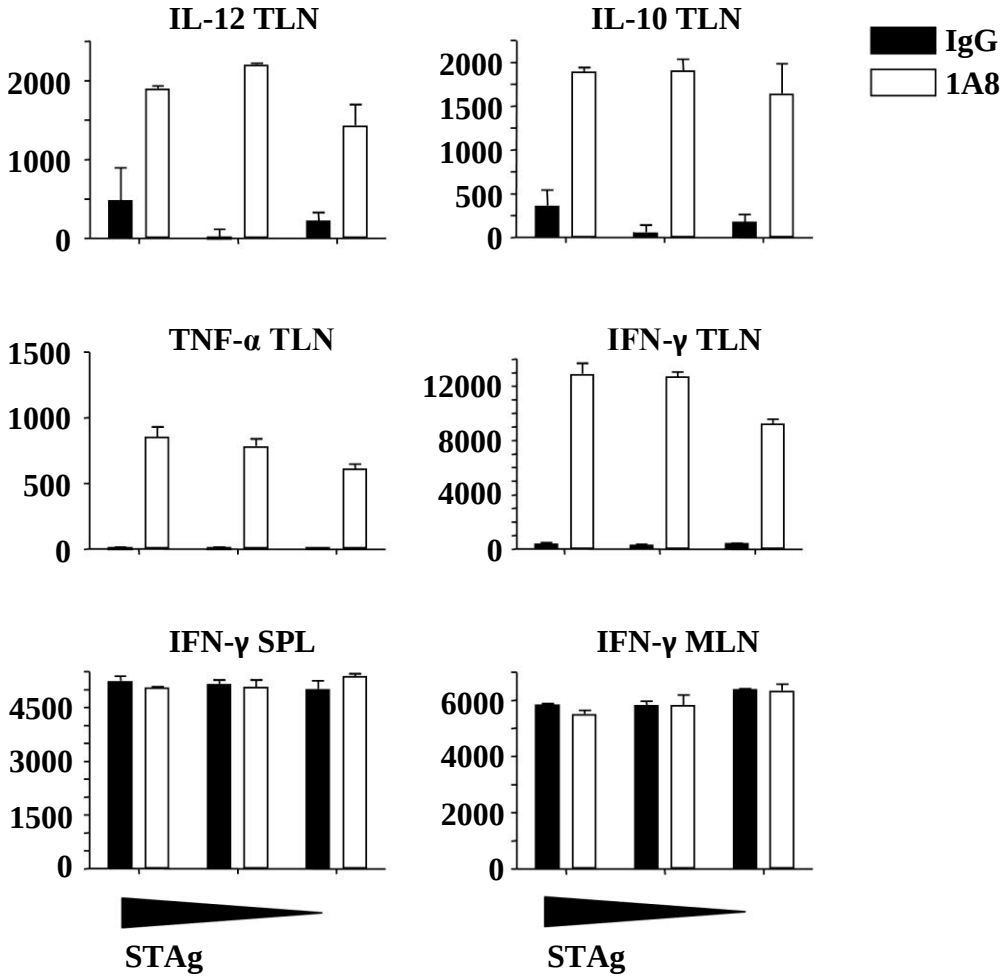

Figure 4.8. Tracheobronchial lymph node restimulation cultures have higher cytokine levels in neutrophil-depleted and infected mice. Single-cell suspensions of tracheobronchial lymph nodes (TLN), spleens (SPL) and mesenteric lymph nodes (MLN) from control-treated or neutrophil-depleted and infected mice were restimulated ex vivo with soluble tachyzoite antigen (STAg) at a range of concentrations (0 μg ml^{-1} - 50 μg ml^{-1}). 24 hr supernatants were collected and tested by ELISA for cytokines. A, IL-12 levels. B, IL-10 levels. C, TNF-α levels. D, IFN-γ levels. E and F show IFN-γ levels in SPL and MLN respectively. All experiments were repeated at least twice with similar results.

$CD4^+$ T cells produce IL-10 and IFN-γ

Given that both BALF and lungs digests of neutrophil-depleted and infected mice have higher percentages of $CD4^+$ T cells, and also given that there are more cytokines produced in these mice as well, we asked whether these $CD4^+$ T cells are the source of cytokine. To address this question, we performed surface staining along with intracellular cytokine staining on cells collected from single-cell suspensions from TLN preparations from control treated and neutrophil-depleted mice. As can be seen in Figure 4.9, and reiterating data from BALF and lung digests, neutrophil-depleted and infected mice had a higher percentage of $CD4^+$ T cells, and a subset of these cells were positive for IL-10 and IFN-γ. Figure 4.9 B and D shows intracellular cytokine staining on $CD4^+$ T cells for IL-10 and IFN-γ respectively, on cells collected from neutrophil-depleted mice, while panels A and C show the same data for control treated samples. $CD8^+$ T cells from depleted and infected mice were also found to produce more cytokine than their counterparts in non-depleted mice (Figure 4.9E). However their percentages were similar in both groups (Figure 4.6B). Intracellular staining for IL-10, IL-17, TNF-α and IFN-γ and surface staining for CD11b and CD11c showed that CD11b cells from depleted mice produced more IL-12 and TNF-α while cytokine levels from CD11c cells were comparable in both groups. It was also notable that the activation status of $CD4^+$ T cells from neutrophil-depleted mice was different from that of $CD4^+$ T cells from control mice. This was evidenced by the expression of CD25 and CD69, where these markers were modestly elevated on $CD4^+$ T cells from neutrophil-depleted mice (Figure 4.10 E and F) compared to $CD4^+$ T cells from control mice (B and C). No differences in CD62L expression on $CD4^+$ T cells was observed between both groups (A and D).

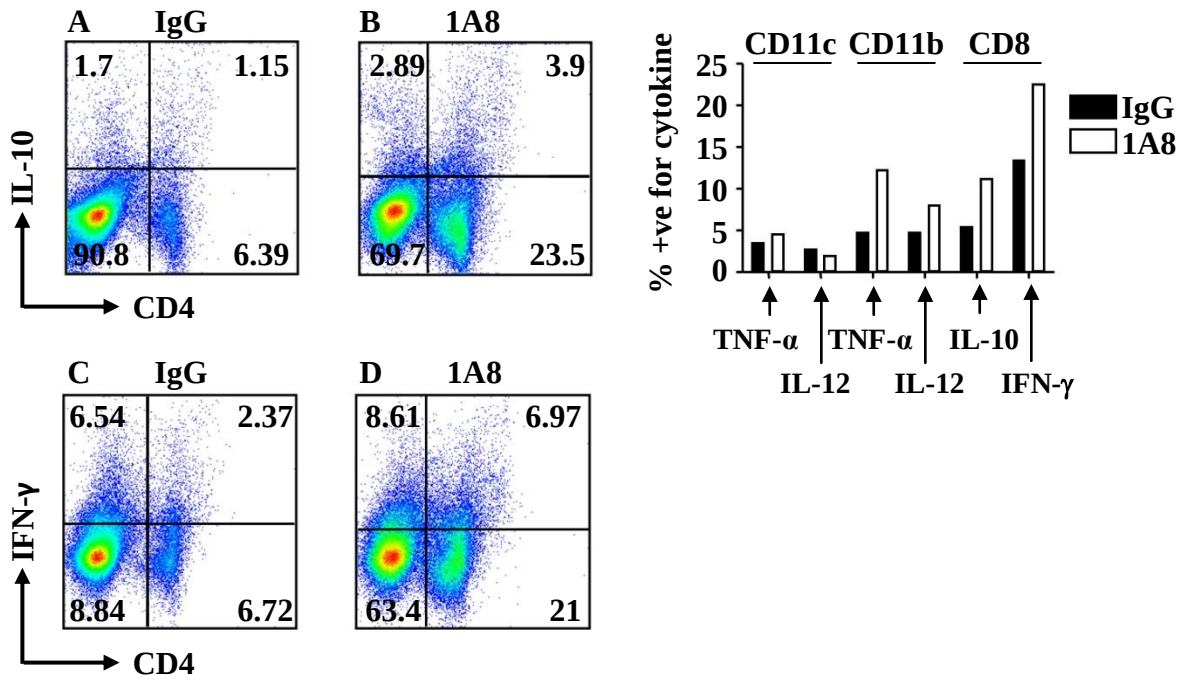

Figure 4.9. T cells are the source of IFN-γ and IL-10 in depleted mice. Tracheobronchial lymph nodes were collected from IgG and 1A8 treated and infected mice and single cells suspensions were collected. Cells were incubated with Golgi plug for 4 hours before staining. Surface staining was performed for CD4, CD8, CD11c and CD11b while intracellular staining was performed for IL-10, IL-12, TNF-α and IFN-γ. A, IL-10 production by cells from IgG treated mice. B, IL-10 production by cells from 1A8 depleted mice. C and D, intracellular IFN-γ staining from IgG and 1A8 treated mice, respectively. E, TNF-α, IL-12, IL-10 and IFN-γ production by CD11c, CD11b and $CD8^+$ T cells. Experiments were repeated at least twice with similar results.

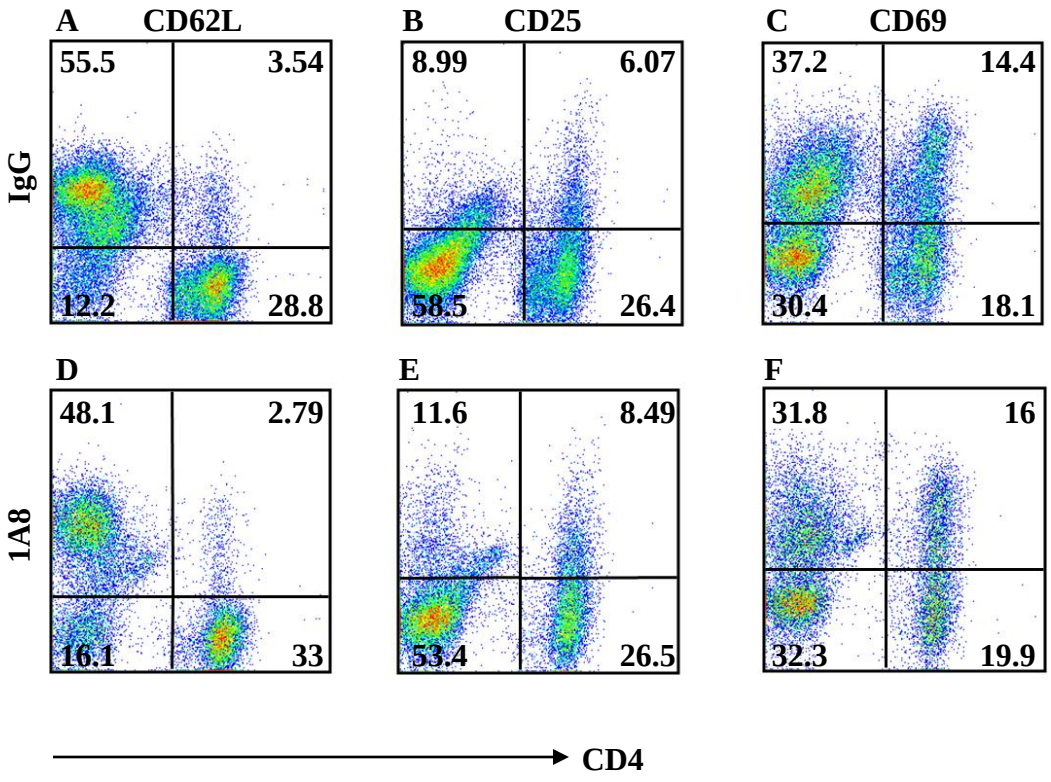

Figure 4.10. CD4$^+$ T cells from tracheobronchial lymph nodes from 1A8 depleted mice display increased levels of activation. Mice were treated with IgG or 1A8 antibodies and infected with 20 cysts of ME49. At day 8 postinfection, tracheobronchial lymph nodes were collected and single cell suspension obtained. Cells were stained for surface markers. Events collected were gated on forward and side scatter. A, B and C, CD62L, CD25 and CD69 staining on CD4$^+$ T cells from control IgG treated mice. D, E and F, CD62L, CD25 and CD69 staining on CD4$^+$ T cells from 1A8 treated mice. Experiments were repeated at least 3 times with similar results.

Dendritic cells from neutrophil depleted mice prime T cells more effectively

Since evidence indicated that $CD4^+$ T cells from neutrophil-depleted mice were greater in number, we asked whether dendritic cells from neutrophil-depleted mice were more effective at activating T cell proliferation. Lungs from infected, control and neutrophil-depleted mice were collected then mechanically and enzymatically treated to obtain single-cell suspensions. Cells were then further subjected to magnetic cell sorting using anti-CD11c beads to isolate dendritic cells. In parallel, OT-II $CD4^+$ T cells were similarly isolated using anti-CD4 magnetic beads from splenocytes of OT-II mice, whose $CD4^+$ T cells are specific for OVA peptide amino acid sequence 323-339. Dendritic cells were incubated with OVA peptide (2 μg ml^{-1}) for 4 hrs at 37°C, then washed extensively and CFSE stained OT-II $CD4^+$ T cells added at a ratio of 10:1. After 4 days in culture, cells were collected and stained for CD4 and analyzed using a flow cytometer for CFSE peak dilution. As can be seen in Figure 4.11, T cells added to dendritic cells isolated from neutrophil-depleted mice exhibit a more pronounced peak dilution indicating that a greater amount of proliferation had occurred.

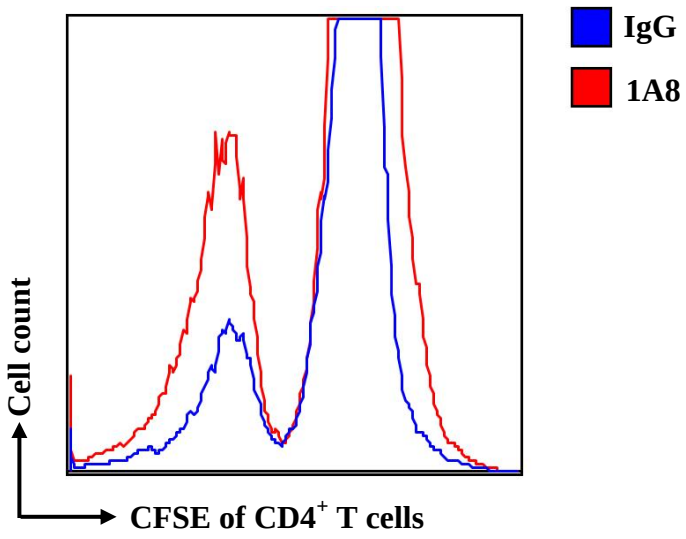

Figure 4.11. Evidence that dendritic cells from 1A8 depleted mice are more potent activators of T cells. Mice were treated with IgG or 1A8 antibodies, and lungs were collected at day 8 postinfection. Dendritic cells purified from lung digests were pulsed with OVA peptide antigen (2µg ml^{-1}) for 4 hours. CFSE stained OT-II $CD4^+$ T cells were added to the dendritic cells at a ratio of 10:1 T cells to DC. 4 days later, cells were collected and analyzed on a flow cytometer for CFSE peak dilution. Blue histogram shows CFSE dilution from $CD4^+$ T cells added to DC isolated from IgG injected mice while the red histogram shows CFSE peak dilution from $CD4^+$ T cells added to DC isolated from 1A8 treated, neutrophil depleted mice.

Discussion

The importance of neutrophils in infection is well established. In addition to being the first cells to arrive at a site of infection or inflammation where they extrude various antimicrobial peptides and molecules, they can function as immunomodulatory cells that can influence an immune response well beyond the innate immunity stage. Here we show that the depletion of neutrophils followed by *Toxoplasma* infection led to rapid host mortality. This neutrophil-specific depletion had no bearing on parasite burden and any pathology in either lymphoid or non-lymphoid tissues with the exception of the lungs. Massive recruitment of cellular foci in the lungs was assayed and determined to be $CD4^+$ T cells. This in turn led to an increased amount of cytokines in depleted and infected mice in the lungs and lung associated draining lymph nodes. This increased production of cytokine was due to overactive $CD4^+$, $CD8^+$ T cells and $CD11b^+$ cells.

A characteristic hallmark of neutrophils is their surface expression of Ly6G molecules (38). The role of neutrophils in vivo can be assayed by the use of Ly6G-specific monoclonal antibody to deplete these cells (32). Our laboratory has previously shown that treating mice with an anti-Gr-1 antibody (Ly6C/G) during a *Toxoplasma* infection leads to impaired immunity characterized by a weaker T_h1 response and uncontrolled tachyzoite replication (36). The Gr-1 surface marker can also be found on a recently described inflammatory monocyte population of cells in the context of *Toxoplasma* infection, as well as on plasmacytoid dendritic cells leading to mixed interpretations of anti-Gr-1 antibody depletion (38-40), thus leading us to re-examine our previously published data with the anti-Gr-1 antibody.

Neutrophil depletion also has major effects on host anti-microbial mechanisms and on the ensuing immune response during other infections. Gr-1^{+} depletion in mice infected with *Leishmania infantum* shows an effect for neutrophils on the early stages of the immune response but no effect late in infection (16). Also, depletion of neutrophils in *Leishmania major* infected mice led to exacerbated footpad lesions (18). However, in some infections neutrophils can contribute to disease. For example, infecting Gr-1^{+} depleted mice with *Plasmodium berghei* ANKA resulted in decreased mortality when compared to non-depleted and infected mice (41, 42). Also, pulmonary infection of Gr-1^{+} depleted mice with *Cryptococcus neoformans* resulted in these mice surviving significantly longer than their non-depleted infected counterparts (43). Various infectious models including *Toxoplasma, Mycobacterium tuberculosis* and *Candida albicans* have highlighted the importance of early depletion of neutrophils when exacerbation of disease is considered, since late depletion of neutrophils had no effect on the outcome of disease (12, 36, 44). Neutrophils can also entrap *Toxoplasma* using neutrophil extracellular traps and a percentage of these parasites are killed when ascertained using the live-cell exclusion dye Sytox green (Chapter 3, Figure 3.5). Although depletion of neutrophils does not seem to affect parasite burden in infected mice, it can very well be that killing and or control mechanisms of the parasite is not limited to neutrophils and their extracellular traps and that the likelihood of redundancy in vivo of cellular functions is very high.

Neutrophils exiting the bone marrow are normally believed to undergo apoptosis within approximately 24 hrs unless otherwise stimulated to prolong their survival by cytokines such as IL-6, G-CSF, TNF-α and IFN-γ (45-48). Since neutrophils carry within their cytoplasmic granules various toxic effector molecules, their apoptosis is followed by a rapid removal from

sites of infection or inflammation to minimize potential tissue damage associated with these molecules. This removal of apoptotic neutrophils is accomplished by phagocytic macrophages, and is important for the resolution of inflammation. Apoptotic neutrophils express specific markers on their surfaces that allows for their recognition by phagocytosing macrophages. For example, mannose-binding lectin (MBL) on macrophages can recognize pathogen-like apoptotic-cell-associated molecular patterns (ACAMPs) on apoptotic neutrophils (49, 50). Also, one of the key "eat me" apoptotic cell markers is phosphatidylserine (PS) which is recognized by PS receptors (50). Evidence also suggests that macrophages that have phagocytosed other apoptotic cells acquire a pro-resolution phenotype, thus reducing the inflammatory response. (2, 51-54). This is in line with our data, where we hypothesize that in the absence of neutrophils (Ly6G treated mice), phagocytic cells are more "pro-inflammatory" due to the lack of apoptotic neutrophils in the milieu (Figure 4.12). This may explain our data showing that dendritic cells are able to present antigen better and more efficiently (Figure 4.11), which in turn may explain the increased number of $CD4^+$ T cells (Figure 4.6). Also, an increased pro-inflammatory state at sites of infection can be expected to lead to activated and inflammatory phagocytes providing a possible explanation for our data showing more cytokine production in $CD11b^+$ cells. Since the majority of IL-10 and INF-γ produced are coming from $CD4^+$ T cells and $CD8^+$ T cells, it would be interesting to know how depletions of T cells would affect onset of death in neutrophil-depleted animals, an area of research currently being investigated in our laboratory. Taken together, our data suggest a model in which mice depleted of neutrophils succumb to *Toxoplasma* infection due to an unchecked and uncontrolled inflammatory response.

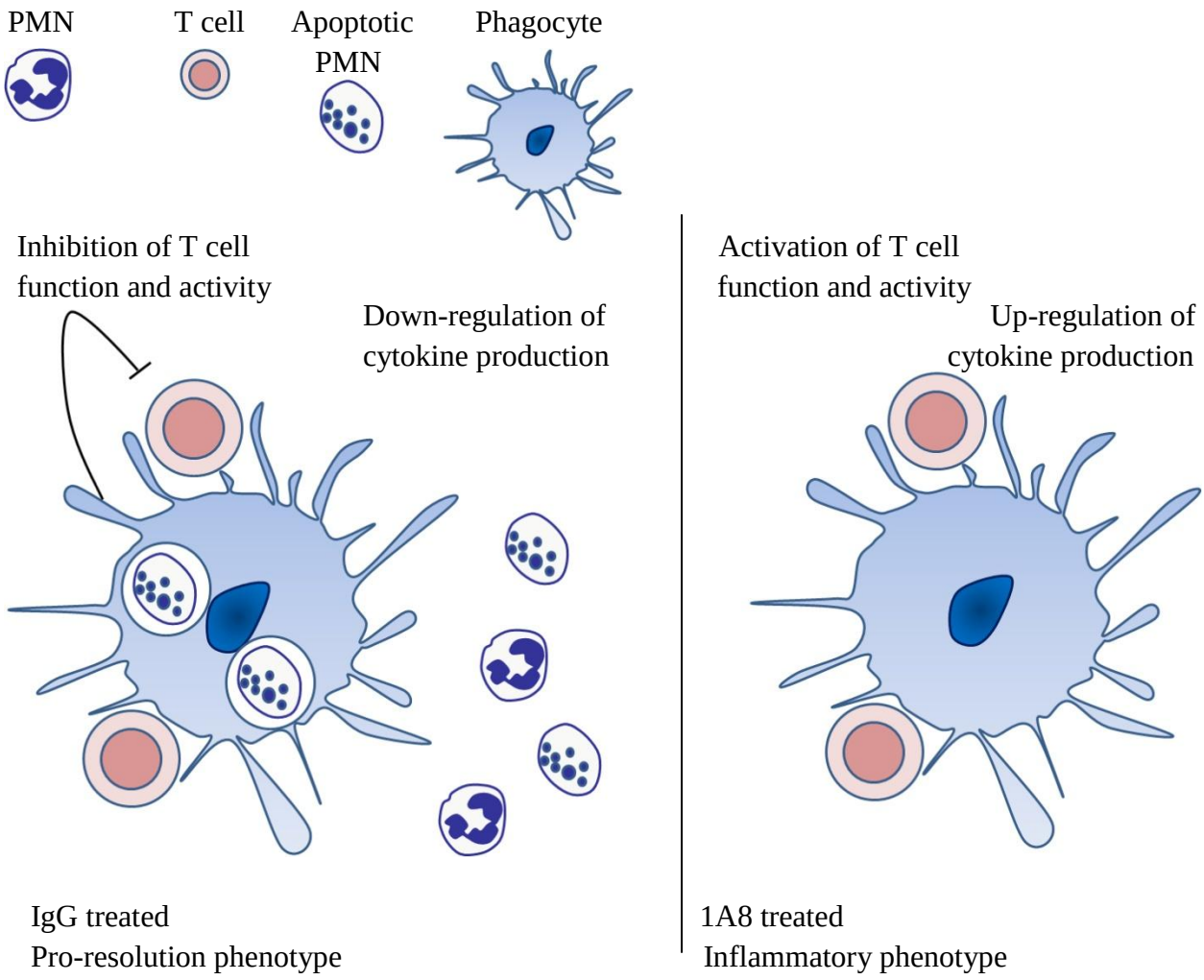

Figure 4.12. Phagocytes from neutrophil depleted and infected mice are more pro-inflammatory. In the absence of neutrophils in 1A8 treated mice, no apoptotic neutrophils are present and as such, phagocytes have a more pro-inflammatory state. This leads to the activation of T cells and the up-regulation of cytokine production. In IgG treated and infected mice, apoptotic neutrophils are abound, and their eventual uptake by phagocytes leads to a pro-resolution activation status of these phagocytes leading to down-regulation of T cell activity and inhibition of cytokine production.

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Chapter 5

DISCUSSION

Summary of findings

Neutrophils play a major role in innate immunity and are usually considered to be short-lived cells with rapid anti-microbial effector functions against various microbial agents. Historically, PMN were viewed as "kamikaze" cells that home to sites of inflammation or infection in response to a chemotactic gradient where they discharge a host of toxic mediators followed immediately by their death through apoptosis (1). However, recent evidence shows that they are far more complicated than previously thought and that they play more nuanced roles in immune responses and immune modulation. An image of neutrophils is emerging as cells that can exert functions well beyond just killing, well beyond their own death, and well beyond the boundaries of innate immunity (2). The data in this thesis support this view.

Data shown in Chapter 2 solidifies the role of neutrophils as antigen presenting cells. In this chapter, I have shown that neutrophils can upregulate molecules required for antigen presentation, namely MHC class II, CD80 and CD86. This upregulation of class II and co-stimulatory molecules on the surface of neutrophils was dependent on contact with T cells, since experiments performed in the presence of a Transwell membrane that prohibits contact between both cell types, abrogated upregulation. After acquisition of antigen processing properties, I showed that PMN can indeed present antigen, by showing that they can uptake, process and present OVA antigen to OVA specific T cells. This antigen presentation led to the proliferation of T cells and to their activation, since T cells exhibited a naïve phenotype before being subjected to PMN antigen presentation and acquired an activated phenotype after antigen presentation. Interestingly, PMN antigen presentation led to differentiation by T cells toward a T_h1 and T_h17 phenotype, evidenced by the production of IFN-γ and IL-17 by PMN/T cell co-

cultures. The cytokine production was entirely by T cells in these co-cultures; intracellular cytokine staining showed no evidence of cytokine production by neutrophils. The production of IL-17 by $CD4^+$ T cells after antigen presentation by neutrophils was found to be both IL-6 and TGF-β independent.

Chapter 3 describes data showing how *Toxoplasma gondii* triggers the release of human and mouse neutrophil extracellular traps. Freshly isolated mouse neutrophils stimulated with PMA in culture produced NETs in a time-dependent fashion. The production of NETs from mouse neutrophils was a controlled process and not due to "cellular bursting", since neutrophils isolated from LYSeGFP mice, that exhibit green fluorescence in every lysozyme positive cell, retained their GFP signal intracellularly. I also showed that *Toxoplasma* can induce NET formation in mouse neutrophils and that this induction was parasite-strain independent, since all three parasite types induced NET formation. Both NET induction in response to PMA and *Toxoplasma* was confirmed using a quantitative dsDNA assay and immunofluorescence microscopy. I also showed that NET induction in response to *Toxoplasma* is invasion independent since the use of cytochalasin D, a drug known to inhibit parasite invasion, did not have an effect on NET induction in neutrophils. Through the use of Sytox green, a live-cell exclusion dye, I was able to show that parasites trapped in NETs are susceptible to killing and that digestion of these NETs with DNAse left the parasites alive. By using differentiated HL-60 cells and freshly isolated human neutrophils from peripheral blood, I was able to demonstrate that human neutrophils also produce NETs in response to *Toxoplasma*. Inhibition of the phosphorylation of ERK using the MEK1/2 inhibitor, U0126, led to decreased NET formation in response to the parasite. The relevance of NET formation in vivo in response to *Toxoplasma* was elucidated in an intranasal

infection model. Lungs from mice infected intranasally with *Toxoplasma* had clear cellular infiltration loci that were shown to be both MPO and *Toxoplasma* positive. Measurement of BALF supernatants for dsDNA showed a massive increase in the amount of extracellular DNA, consistent with NET formation.

Chapter 4 examines the role of neutrophils during a *Toxoplasma* infection. Treatment of mice with the 1A8 antibody (anti-Ly6G) completely depleted neutrophils in vivo. This depletion was monitored by flow cytometry, immunohistochemistry and peripheral blood smears. I found that neutrophil-depleted and infected mice succumb to *Toxoplasma* within 10 days post-infection. Given their sudden death, I decided to find out whether neutrophil-depleted and infected mice had any defect in their control of *Toxoplasma.* To my surprise, I found that neutrophil-depleted and infected mice had similar parasite burdens in all tissues tested when compared to their non-depleted and infected counterparts. Also to my surprise, there was no obvious difference in tissue pathology between both groups in most lymphoid and non-lymphoid organs. However, extreme differences in both pathological and cytokine responses were observed in the lungs of mice. Mice depleted of neutrophils and infected with *Toxoplasma* exhibited an increased amount of cellular infiltration into the lungs. When assayed for cellular content, these infiltrates were determined to be mostly composed of $CD4^+$ T cells. Interestingly, lung digests and BALF contained elevated numbers of B cells and macrophages in addition to the elevated numbers of $CD4^+$ T cells. Given this increased number of cells, I decided to examine the cytokine profiles in these mice. Supernatants from BALF of depleted and infected mice showed increased levels of TNF-α and IL-12. Also, culture supernatants from STAg restimulated tracheobronchial lymph node cells exhibited elevated levels of IL-10, IL-12, TNF-α and IFN-γ. Interestingly, this

differential elevated cytokine response was shown to be localized to the lung, since restimulation assays using cells from spleens and mesenteric lymph nodes showed no difference between neutrophil-depleted and non-depleted and infected mice. Intracellular cytokine staining showed that the $CD4^+$ and $CD8^+$ T cells are the source IFN-γ and IL-10. Similar staining performed on CD11b positive cells showed that they are the source of IL-12 and TNF-α. Flow cytometric analysis I performed showed that $CD4^+$ T cells isolated from tracheobronchial lymph nodes in depleted mice display increased levels of activation. I also found evidence for increased dendritic cell activation in the lungs of depleted and infected mice evidenced by their increased ability to induce more robust T cell proliferation.

Future directions and unanswered questions

Evidence for a role of neutrophils as antigen presenting cells had been reported before. Patients with chronic autoimmune disorders, rheumatoid arthritis and Wegner's granulomatosis, have been shown to have neutrophils that are elevated for surface expression of MHC class II. Neutrophils from some of these patients have been also shown to have an upregulation of co-stimulatory molecules, especially after cytokine stimulation (3-5). A more recent report showed that neutrophils can present OVA peptide-antigen (aa 323-339) to $CD4^+$ OVA specific T cells (6).

Data presented in Chapter 2 confirms this antigen presentation ability of neutrophils while at the same time is distinguished by the fact that it is the first report to show that neutrophils also have the ability to process whole antigen and present it to T cells. Also, it is the first report to show that neutrophil antigen presentation skews responding T cells towards a T_h1 and T_h17 profile.

What makes my Th17 skewing data interesting is the fact that it does not appear to require TGF-β or IL-6. This is an area that needs to be investigated further as it would be very interesting to identify the neutrophil factors driving T_h17 differentiation. It also remains to be elucidated as to what role if any neutrophils play in antigen presentation in vivo in light of their ability to do so in vitro. Given the wide-ranging ability of neutrophils to perform various functions, it would be interesting to know whether their APC abilities are needed in vivo in the context of disease if other APC, such as DC, are present and sufficiently priming T cells. A mouse model engineered to lack MHC molecules in PMN would be very useful here.

In addition to their role in antigen presentation, ample evidence exists now showing that neutrophils can trap and/or kill pathogens by release of extracellular nuclear DNA (7). NETs formed as a result of the newly described neutrophil cell death process, NETosis, can trap and kill pathogens extracellularly (8). In Chapter 3 I show that NETs are formed in both mouse and human neutrophils as a result of *Toxoplasma* infection and that NET formation leads to the entrapment of *Toxoplasma* and that some parasites are killed by NETs. This is the first report to show that NETs are formed in response to *Toxoplasma* and that neutrophils can play a role in the extracellular killing and control of the parasite. The entrapment by NETs led to the killing of a certain percentage of the parasites (~25%) raising the possibility that the major role of NETs in the control of *Toxoplasma gondii* might not necessarily be the killing of the parasite, but more so the prohibiting of its spread. Since *Toxoplasma* is an obligate intracellular parasite, trapping it outside a cell will eventually lead to its demise. It remains to be elucidated whether entrapment by NETs prohibits infection of neighboring cells, in other words, whether the parasite can escape NET formation and continue to invade a host cell. This is a crucial question, given that I did not

observe complete parasite killing. Also, given that NET formation in response to *Toxoplasma* was not dependent on parasite invasion and that soluble tachyzoite antigen induced some NET formation, it is very likely that the parasitic trigger for NET formation is a secreted product. In experiments using cytochalasin D to block active invasion, I continued to observe NET formation. However, I cannot draw conclusions as to whether intracellularly infected neutrophils are still capable of undergoing NETosis. Although I saw no difference in NET formation in response to the parasite with or without cytochalasin D, it remains unclear whether NETosis observed was by infected or non-infected neutrophils since in the absence of cytochalasin D, not all cells are infected. Answering this question will help elucidate whether the parasite has mechanisms that can inhibit or interfere with NET formation and/or whether neutrophils themselves can carry out steps to counteract parasite manipulation of host mechanisms.

Illuminating the role NETs can play in the control of *Toxoplasma* in vivo is an area that should be further investigated. *Toxoplasma* is well-known for the ability to infect any nucleated cell and by doing so can escape the extracellular milieu where NETs would have their parasite-destructive effects. Since microbial infections trigger robust NET formation by neutrophils it would be interesting to know what role if any NETs play in contribution to disease and inflammation, especially in light of evidence that NETs are implicated in various autoimmune conditions. How NET formation contributes to parasite control or contributes to overall disease by possibly exacerbating inflammation is an area that needs further questioning.

Conflicting reports have emerged over the role of neutrophils during *Toxoplasma* infection owing to the fact that historically anti-Ly6C/G (Gr-1) antibody had been the classical antibody of

choice for rendering mice neutropenic (9-12). This surface marker has since been described to occur on other cells as well, questioning the validity of data using anti-Gr-1 monoclonal antibody as the antibody of choice for neutrophil depletions. A more recent antibody, so far shown to be only neutrophil specific, is being used as the new gold standard for in vivo depletions (13). Using this antibody, I depleted neutrophils in vivo and infected with *Toxoplasma* resulting in lethal effects.

Data shown in Chapter 4 demonstrates that in the absence of neutrophils, infection leads to a dysregulated immune response resulting in detrimental effects to the host. Mice depleted and infected exhibited abnormal pulmonary pathology characterized by dysregulated cytokine production and increased cellular infiltration. In these experiments, I observed increased cytokine production from cells isolated from neutrophil depleted and infected mice. Increased cytokine activity was attributed to $CD4^+$ and $CD8^+$ T cells as well as $CD11b^+$ cells. It is possible that this dysregulated inflammatory response is due to the fact that in the absence of neutrophils at sites of inflammation, an immune response is not resolved as it normally would be due to lack of apoptotic neutrophils. During the normal process of resolution of inflammation, phagocytes take up apoptotic neutrophils and then undergo a down-modulatory shift, helping to resolve the immune response. In my model, lack of neutrophils led to an increased dendritic cell activation status as determined by increased T cell proliferation and increased cytokine production. This conclusion is based on DC:T cell proliferation studies, and no experiments were performed to assay for the activation status of DC between both depleted and non-depleted and infected groups. This is an issue that needs to be answered in the future. Also, since lung pathology seems to be mostly dependent on an infiltration of T cells, and increased cytokine production from T

cells is seen in depleted and infected mice, it would be very informative to co-deplete mice of both neutrophils and T cells to determine if an amelioration of lung pathology occurs or if host survival is lengthened.

My depletion of neutrophils in vivo had no effect on *Toxoplasma* tissue burden in almost all tissues tested. This seems in apparent contrast to my NET data in Chapter 3 where neutrophils extruded traps that entangled *Toxoplasma* and led to some parasite killing. Although this may seem paradoxical, it is possible that by invading cells, the parasite escapes the effects of extracellular traps. Also, while parasite number was not apparently affected by neutrophil depletion, these studies were carried out in mice. Therefore, it is possible in other host species, NETs are important in controlling parasite number. In this regard, I found that human neutrophils were more responsive to NET formation than mouse neutrophils.

Future work is required to address all the issues raised above. It is however very clear, that neutrophils are not just scavenging cells that travel to sites of infection and inflammation to destroy and kill only. Data generated by myself and by others show that neutrophils are major immunoregulatory cells that can influence and initiate immune responses. PMN are cells that are capable of multiple functions and are emerging as regulators of immune responses that play important roles in inflammation.

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